THE
BIOLOGICAL BULLETIN

Woods Hole, Mass.
PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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Vol. LIX, No. 1 August, 1930

THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

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FORTY-SECOND YEAR

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 12 o'clock noon, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years. There shall be thirty-two Trustees thus chosen divided into four classes, each to serve four years, and in addition there shall be two

groups of Trustees as follows: (*a*) Trustees *ex officio,* who shall be the President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer and the Clerk; (*b*) Trustees Emeriti, who shall be elected from the Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next annual meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee Emeritus for life. The Trustees *ex officio* and Emeriti shall each have the same right to vote as the regular Trustees.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

II. Special meetings of the members may be called by the Trustees to be held in Boston or in Woods Hole at such time and place as may be designated.

III. The Clerk shall give notice of meetings of the members by publication in some daily newspaper published in Boston at least fifteen days before such meeting, and in case of a special meeting the notice shall state the purpose for which it is called.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. The Trustees shall have the control and management of the affairs of the Corporation; they shall present a report of its condition at every annual meeting; they shall elect one of their number President of the Corporation who shall also be Chairman of the Board of Trustees; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

VI. Meetings of the Trustees shall be called by the President, or by any two Trustees, and the Secretary shall give notice thereof by written or printed notice sent to each Trustee by mail, postpaid. Seven Trustees shall constitute a quorum for the transaction of business. The Board of Trustees shall have power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient.

VII. The accounts of the Treasurer shall be audited annually by a certified public accountant.

VIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

IX. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

X. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

IV. THE REPORT OF THE TREASURER

To the Trustees of the Marine Biological Laboratory:

Gentlemen: Herewith is submitted my report as Treasurer of the Marine Biological Laboratory.

The accounts have been audited by Seamans, Stetson and Tuttle, certified public accountants. A copy of their report is on file at the Laboratory and is open to inspection by members of the Corporation.

The book value of the General Endowment Fund in the hands of the Central Hanover Bank and Trust Company (of New York) as Trustees, was $908,145 in securities and $804.50 in cash.

A new trust known as the Library Endowment Fund was created by the gift on December 14th, of $200,000 from the General Education Board, and is also in the hands of the Central Hanover Bank and Trust Company. At the end of the year it was invested in bonds to the extent of $80,865 and the cash balance was $119,135. This was shortly thereafter invested to yield about five per cent (5%).

At the end of the year the Lucretia Crocker Fund consisted of securities of the book value of $4,442.46 and cash of $537.20.

The Ida H. Hyde Scholarship Fund, which consisted of $2,000 in a mortgage participation and interest, has been, with the consent of all parties, returned to Dr. Hyde, who has turned the Fund over to the University of Kansas for the benefit of whose students the Fund was established.

The Bio Club Scholarship Fund consisted of a $2,000 participation in a mortgage and cash of $23.28, and the Reynold A. Spaeth Memorial Lecture Fund consisted of a $3,000 participation in a mortgage and cash of $1.83.

The Retirement Fund at the end of the year consisted of $13,000 in mortgage participations and cash of $14.27. The income is approximately $650, the annual addition (5% of the permanent annual payroll) amounts to about $2,600 and the pension payments now authorized are at the rate of $720 annually.

The land, building, equipment and library, excluding the Devil's Lane and Gansett property, represented an investment of $1,600,352.93 —less depreciation of $208,722.91—or a net amount of $1,391,630.02.

Current income for the year was exceeded by expenditures including depreciation by $855.33.

Over $20,000 was expended from current funds on buildings, equipment, books and reduction of mortgages.

At the end of the year the Laboratory owed less than $1,500 in accounts payable, and $27,000 on mortgages.

Following is the Balance Sheet at the end of the year, and the condensed statement of income and outgo for the year, also the surplus account.

EXHIBIT A

MARINE BIOLOGICAL LABORATORY BALANCE SHEET,

DECEMBER 31, 1929

Assets

Endowment Assets and Equities:			
Securities and Cash in Hands of Central Hanover Bank & Trust Company (of New York) Trustee—Schedules I-a and I-b		$1,108,949.50	
Securities and Cash—Minor Funds—Schedule II		12,049.77	$1,120,999.27
Plant Assets:			
Land—Schedule IV	$ 115,553.05		
Buildings—Schedule IV	1,206,242.06		
Equipment—Schedule IV	149,958.05		
Library—Schedule IV	128,599.77	$1,600,352.93	
Less Reserve for Depreciation		208,722.91	
		$1,391,630.02	
Cash in Dormitory Building Funds		1,451.76	$1,393,081.78
Current Assets:			
Cash		$ 17,144.45	
Accounts—Receivable		17,648.13	
Inventories:			
Supply Department	$ 35,850.39		
Biological Bulletin	7,461.00	43,311.39	
Investments:			
Devil's Lane Property	$ 36,260.01		
Gansett Property	2,076.30		
Stock in General Biological Supply House, Inc.	12,700.00		
Retirement Fund Assets	13,014.27	64,050.58	
Prepaid Insurance		3,986.80	
Items in Suspense		256.42	146,397.77

Liabilities

Endowment Funds:			
General Endowment Funds—Schedule III		$1,108,949.50	
Minor Endowment Funds—Schedule III		12,049.77	$1,120,999.27
Plant Funds:			
Donations and Gifts—Schedule III		$1,019,840.41	
Other Investments in Plant from Gifts and Current Funds		371,241.37	
		$1,391,081.78	
Mortgage, Danchakoff Estate		2,000.00	$1,393,081.78

Current Liabilities and Surplus:		
Mortgage, Devil's Lane Property	$ 25,000.00	
Accounts—Payable	1,464.48	
	$ 26,464.48	
Current Surplus—Exhibit C	119,933.29	146,397.77

EXHIBIT B

Marine Biological Laboratory Income and Expense, Year Ended December 31, 1929

	Total		Net	
	Expense	Income	Expense	Income
Income, General Endowment Fund		$ 46,549.09		$ 46,549.09
Income, Fund for Library, etc.	793.95	4,582.20		3,788.25
Gifts (See Current Surplus)				
Instruction	7,560.82	9,400.00		1,839.18
Research	4,073.91	16,625.00		12,551.09
Biological Bulletin and Membership Dues	6,288.58	8,025.93		1,737.35
Supply Department—Schedule V	52,611.28	62,056.83		9,445.55
Mess, Schedule VI	33,038.05	34,581.67		1,543.62
Dormitories, Schedule VII	31,386.60	13,754.81	17,631.79	
(Interest and Depreciation charged to above three Depts. See Schedules V, VI, and VII)	35,366.31			35,366.31
Dividends on Stock, General Biological Supply House, Inc.		2,540.00		2,540.00
Rent, Danchakoff Cottages	430.53	900.00		469.47
Rent, Microscopes		335.00		335.00
Rent, Garage, Railway, etc.		163.90		163.90
Rent, Newman Cottage	157.03	150.00	7.03	
Sale of Duplicate Library Sets		475.00		475.00
Interest on Bank Balances		267.63		267.63
Sundry Items		1.85		1.85
Maintenance of Plant:				
New Laboratory Expense	15,234.18		15,234.18	
Maintenance, Buildings and Grounds	10,399.72		10,399.72	
Chemical and Special Apparatus	9,144.10		9,144.10	
Library Department Expenses	8,894.07		8,894.07	
Carpenter Department Expenses	1,587.80		1,587.80	
Truck Expenses	800.47		800.47	
Sundry Expenses	148.18		148.18	
Bar Neck Property Expenses	381.00		381.00	
Evening Lectures	120.09		120.09	

Workmen's Compensation Insurance	598.23		598.23	
Janitor's House	289.42		289.42	
Pumping Station	267.49		267.49	
General Expenses:				
Administration Expenses	13,649.48		13,649.48	
Interest on Loans	259.00		259.00	
Endowment Trust Fund	787.50		787.50	
Bad Debts	523.96		523.96	
Contribution for Research, Naples Zoological Station	250.00		250.00	
Reserve for Depreciation	36,955.11		36,955.11	
Excess of Expense over Income carried to Current Surplus—Exhibit C		855.33		855.33
	$201,264.24	$201,264.24	$117,928.62	$117,928.62

EXHIBIT C

MARINE BIOLOGICAL LABORATORY, CURRENT SURPLUS ACCOUNT

YEAR ENDED DECEMBER 31, 1929

Balance, January 1, 1929		$100,637.23
Add:		
Gifts Received		
From General Education Board for Purchase of Books for Library		10,000.00
From Dr. Frank R. Lillie for Grading, Planting Walks, etc., around Laboratory Buildings		1,950.00
Income from Retirement Fund		305.04
Reserve for Depreciation charged to Plant Funds		36,955.11
		$149,847.38
Deduct:		
Payments from Current Funds during Year for Plant Assets as shown in Schedule IV,		
Buildings	$ 1,094.23	
Equipment	5,638.67	
Library Books, etc.	9,218.54	
	$15,951.44	
Payments from above Gifts charged to Plant Assets, General Education Board, Purchase of Books	6,657.32	
Dr. Frank R. Lillie, Grading, etc.	1,950.00	
Payments on Mortgages, Danchakoff Estate	4,500.00	
Balance of Income-and-Expense Account—Exhibit B	855.33	29,914.09
Balance, December 31, 1929—Exhibit A		$119,933.29

Respectfully submitted,

LAWRASON RIGGS, JR.,
Treasurer.

V. THE REPORT OF THE LIBRARIAN

The close of the year 1929 marks a new period for the Library. The $50,000 presented by the General Education Board has become an integral part of the Library in the form of serial and monograph sets. A Library Endowment Fund of $200,000 by the same donor has been invested by the Treasurer of the Laboratory, the interest to form part of the annual Library budget. Besides this, and for the year 1929, a sum of $4,582.20, interest accumulated between the gift date and the actual transfer to the Laboratory, is now practically covered by outstanding orders.

With a steady income assured by this endowment fund, it becomes possible to plan a yearly budget, proportioned approximately as follows:

Books	$ 300
Serials	5,000
Binding	1,400
Back Sets (Serials and Reprints)	8,200
Express	300
Supplies	500
Salaries	8,300
Total	$24,000

The most notable feature brought out in contrast to past years is that current serials heretofore confined strictly to the Laboratory budget and not benefited by the special gift of the General Education Board will now share largely in the interest from the permanent endowment, and will form a total item of expenditure just about equal each year to the sum spent on back sets.

For convenience of future reference, etc., we would like to print here our full report of expenditures of the $50,000 gift just as these were reported each of the four years to the General Education Board. But the score of pages entailed scarcely justifies this procedure and the list may be consulted at any time in the Library files.

Besides that part of the above-mentioned figures for 1929, the expenditures: Books, $300.43; Serials, $4,787.19; Binding, $1,232.36; Express, $253.23; Supplies, $558.34; Salaries, $8124.18; total, $15,-253.73, show an overrun of the budget ($14,000) amounting to $1,-253.73, in accordance with the expected (see Report of the Library, 1927 and 1928). A sum of $929 from the sale of accumulated duplicates, etc., almost covers this and eventually (early in 1930), the Library will cancel the remaining deficit by the same means.

The Library starts on its new period with 23,455 bound serials; 4,855 books; and 58,658 reprints. A comparison with the 1928 report will give the 1929 actual figures of accretion. The serials now show

1,537 titles; of these 985 are received currently: 322 by subscription; 420 by exchange; and 253 by gift.

The Librarian wishes to acknowledge in the name of the Marine Biological Laboratory the following gifts to the Library:

From the authors: Addison, W. H. F. (Editor): *Piersol's Normal Histology;* Conn, H. J.: *Biological Stains;* Correns, Carl: *Gesammelte Abhandlungen zur Vererbungswissenschaft aus Periodischen Schriften, 1899–1924;* Goldschmidt, Richard: *Der Mendelismus, in Elementarer Darstellung;* Hance, Robert T.: *The Machines We Are;* Heilbrunn, L. V.: *The Colloid Chemistry of Protoplasm;* Lumière, Auguste: *Le Cancer, Maladie des Cicatrices;* Lumière, Auguste: *Le Problème de l'Anaphylaxie;* Lumière, Auguste: *La Vie, la Maladie et la Mort;* Patten, Bradley M.: *The Early Embryology of the Chick;* Schafer, Edward Sharpey: *History of the Physiological Society during its First Fifty Years, 1876–1926;* Whiting, P. W.: *A Series of Eight Radio Talks on Heredity and Human Problems;* Williams, Samuel Howard: *The Mammals of Pennsylvania;* Woodruff, Lorande L.: *Foundations of Biology.*

From the publishers:

Baillière, Tindall & Cox; Gaskell, Augusta: *What is Life?*

Thos. Y. Crowell Co.; Papez, James W.: *Comparative Neurology;* Wheeler, Raymond H.: *The Science of Psychology.*

Ginn and Co.; Edison, Oskar E. and Norris, F. W.: *Electrical Engineering Laboratory Practice;* Pierce, B. O.: *Short Tables of Integrals.*

Harvard University Press; Banks, Nathan and Myers, J. G.: *Studies on Cuban Insects, I.*

Paul B. Hoeber; Gross, Louis: *The Blood Supply to the Heart;* McClung, C. E. (Editor): *Handbook of Microscopical Technique;* Roth, Irving R.: *Cardiac Arrhythmias;* Stone, Willard J.: *Blood Chemistry, Colorimetric Methods;* Wright, Wilhelmine G.: *Muscle Function.*

Johns Hopkins Press; Camerson, Jenks: *The Bureau of Biological Survey;* Powell, Fred W.: *The Bureau of Plant Industry.*

Alfred A. Knopf, Inc.; Knickerbocker, Wm. S. (Editor): *Classics of Modern Science;* Nordenskiöld, Erik: *The History of Biology;* Wheeler, Wm. M.: *Foibles of Insects and Men.*

Lea & Febiger; Faust, Ernest Caroll: *Human Helminthology;* Kuntz, Albert: *The Autonomic Nervous System.*

McGraw-Hill Book Co.; Babcock, Ernest B. and Collins, J. L.: *Genetics, Laboratory Manual;* Daniels, F.: *Mathematical Preparation for Physical Chemistry;* Gäumann, Ernst A.: *Comparative Morphology of Fungi;* Goodwin, H. M.: *Precision of Measurements and Graphical Methods;* Joffe, Abram F.: *Physics of Crystals;* Knowlton, A. A.:

Physics for College Students; Metcalf, C. L. and Flint, W. P.: *Destructive and Useful Insects; Their Habits and Control;* Richtmyer, F. K.: *Introduction to Modern Physics;* Weaver, John E. and Bruner, Wm. E.: *Root Development of Vegetable Crops;* Weiser, Harry B.: *The Colloidal Salts.*

Macmillan Co.; Carpenter, Kathleen E.: *Life in Inland Waters with Especial Reference to Animals;* Coghill, G. E.: *Anatomy and the Problem of Behaviour;* Darwin, Charles: *The Origin of Species;* deBeer, G. R.: *Vertebrate Zoology;* Holmes, Samuel J.: *The Biology of the Frog;* Lindsey, Arthur Ward: *Textbook of Evolution and Genetics;* Walter, H. E.: *Biology of the Vertebrates;* Walter, H. E.: *Genetics;* Washburn, M. F.: *Animal Mind.*

Wm. Morrow & Co.; Stanford, Alfred: *Invitation to Danger.*

Museo Nacional de Ciencias National, Madrid: Lozano, Luis Rey: *Peces (Fauna Ibérica), vol. 1.*

G. P. Putnam's Son, Ltd.; Anthony, H. E.: *The Field Book of North American Mammals;* Breder, Charles M., Jr.: *Field Book of Marine Fishes of the Atlantic Coast.*

W. B. Saunders Co.; Dorland, W. A. Newman: *The American Illustrated Medical Dictionary;* Granger, Frank Butler: *Physical Therapeutic Technic;* Jordan, Edwin O.: *A Text-book of General Bacteriology;* Lusk, Graham: *The Elements of the Science of Nutrition;* Stiles, Percy G.: *Human Physiology;* Willius, Frederick A.: *Clinical Electrocardiograms, Their Interpretation and Significance.*

Smith College; Funkhouser, W. D.: *General Catalogue of the Hemiptera, Fasc. I.*

Charles C. Thomas, Publisher; Ewing, Henry Ellsworth: *A Manual of External Parasites;* Friedmann, Herbert: *The Cowbirds;* Needham, James G.: *Elementary Lessons on Insects;* Needham, James G. and Heywood, H. B.: *A Handbook of the Dragonflies of North America.*

University of California Press: Daniel, J. Frank: *The Elasmobranch Fishes;* Grinnell, Joseph & Storer, T. I.: *Animal Life in the Yosemite;* Grinnell, Joseph; Brayant, H. C., and Storer, T. I.: *The Game Birds of California;* Russell, E. J.: *Plant Nutrition and Crop Production.*

University of Chicago Press; Choulant, Ludwig: *History and Bibliography of Anatomy Illustration;* Michelson, A. A.: *Studies in Optics;* Morse, Jared Kirtland: *Bibliography of Crystal Structure;* Ornstein, Martha; *The Rôle of Scientific Societies in the Seventeenth Century.*

University of Colorado Press; Cockerell, T. D. A.: *Zoölogy of Colorado;* George, R. D.: *Geology and Natural Resources of Colorado;* Ramaley, F.: *Colorado Plant Life.*

D. Van Nostrand Co.; Robbins, Wm. J. and Rickett, Harold W.:

Botany, a Textbook for College and University Students; Robbins, W. J. and Rickett, Harold W.: *Laboratory Instructions for General Botany.*

Victor X-Ray Corporation; Jerman: *Modern X-Ray Technic.*

John Wiley & Sons; Pearse, A. S. and Hall, F. G.: *Homoiothermism, the Origin of Warm-Blooded Vertebrates;* Shumway, Waldo: *Vertebrate Embryology.*

Williams and Wilkins Co.; Bailey, Vernon: *Animal Life of the Carlsbad Cavern;* Brasch, Frederick E.: *Sir Isaac Newton, 1727–1927;* Brouwer, B.: *Anatomical, Phylogenetical and Clinical Studies on the Central Nervous System.*

Wisconsin Academy of Science; Baker, Frank C.: *Fresh Water Mollusca of Wisconsin.*

William Wood & Co.; Bailey, R. B. and Miller, A. M.: *Textbook of Embryology;* Stedman: *Medical Dictionary.*

Yale University Press; Brown, William: *Science and Personality;* Chittenden, Russell H.: *History of the Sheffield Science School of Yale University, 1846–1922;* Crawford, Albert Beecher: *Incentives to Study;* Eddington, A. S.: *Stars and Atoms;* Henderson, L. J.: *Blood, A Study in General Physiology;* Henderson, Yandell and Davie, M. R.: *Incomes and Living Costs of a University Faculty;* Krogh, August: *Anatomy and Phsyiology of Capillaries;* Millikan, Robert A.: *Evolution in Science and Religion;* Morgan, Thomas Hunt: *The Theory of the Gene;* Peake, Harold and Fleure, Herbert John: *The Corridors of Time, I–V;* Robbins, Wm. Jacob and Samuel Brody, et al: *Growth;* Sumner, W. G. and Keller, A. G.: *The Science of Society;* Wilm, E. C.: *The Theories of Instinct;* Wilson, Edmund B.: *The Physical Basis of Life.*

The Library also desires to acknowledge the gift of two hundred reprints from Dr. H. McE. Knower; papers from Dr. Fauré-Fremiet, which include the works of Balbianai, Henneguy, Thelohon, Lecauller, and Loyez; and a collection of 131 books from Mr. Ware Cattell; also a collection of reprints on Tunicates and Ascidians, from Dr. Harold S. Colton.

VI. THE REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen: I beg to submit herewith a report of the forty-second session of the Marine Biological Laboratory for the year 1929.

1. *Attendance.* An inspection of the Tabular View of Attendance for the years 1925 to 1929, inclusive, on page 33 will show that since

1927, when crowding of the laboratory buildings and particularly of the Mess during the middle of the summer first became a serious problem, there have been no great changes in the total attendance, the figures for 1927, 1928 and 1929 for investigators and students together being respectively, 434, 454 and 444. It may be noted, however, that although the total for 1929 was slightly less than that for 1928, there was at the same time an increase of 6 in the number of investigators over the previous high record of 323 in 1928. The experience of the past three years indicates that with the present facilities of the Laboratory a materially increased registration will be impossible in the future without an extension of the active season on one or both sides of the present crowded period covering chiefly the months of July and August. How to bring about such an extension is a problem the solution of which will call for the coöperation of all of the workers associated with the Laboratory.

While the total attendance for 1929 was approximately the same as that for 1928, a considerable improvement in the distribution of the attendance was brought about during the year covered by this report by a re-arrangement of the courses of instruction in such a way as to spread them over a period of twelve weeks instead of six as in the past. In 1929 the courses in Botany, Embryology and Physiology were given between June 19 and July 30 inclusive and those in Invertebrate Zoölogy and Protozoölogy between August 1 and September 11, inclusive. Not only did this arrangement reduce the number of students in attendance at any one time during the crowded season, but it tended to emphasize to members of the teaching staffs and to others the desirability of making August 1 a point of division of the season for those unable to be in attendance during the entire summer. An additional advantage of the new arrangement was the freeing of one of the large classrooms and its conversion into nine private laboratories of a desirable type not previously available for investigators.

The results of this change are most strikingly shown by the following figures for attendance at the Mess on selected days for 1928 and 1929.

		1928	1929
May	30	0	0
June	10	63	73
"	20	157	228
"	30	419	304
July	10	484	386
"	20	470	387
"	30	444	386

August	10	335	431
"	20	323	401
"	30	265	357
September	10	124	234
"	20	14	68
"	30	0	0

It will be noted that the sharp peak which characterized the Mess attendance for 1928 and to a somewhat lesser extent that for previous years is greatly flattened in 1929. It may be mentioned that the reduction of more than 11 per cent in the maximum daily attendance (438 on August 7 in 1929 as compared with 495 on July 14, 1928) was accomplished in spite of an increase of 7 per cent in the total number of meals served during the season (33,633 in 1929 as compared with 31,555 in 1928). Furthermore, in 1929 the length of the period during which more than 400 persons were served daily was only 22 days, while in 1928 it was 40 days. It is believed that some slight disadvantages of the new arrangement of the courses are more than compensated for by the reduction in crowding indicated by the above figures.

That the new arrangement of the courses was also not without an effect on the distribution of the attendance by investigators is shown by the following figures for four months each of the three years, 1927, 1928 and 1929, which indicate very clearly a flattening and a spreading of the undesirable peak of attendance for the year last mentioned:

		1927	1928	1929
May	30	7	15	9
June	10	50	64	55
"	20	114	140	139
"	30	212	240	197
July	10	247	281	238
"	20	247	282	242
"	30	245	272	249
August	10	234	250	256
"	20	208	226	243
"	30	168	183	220
September	10	110	112	157
"	20	50	43	59
"	30	12	14	14

2. *The Report of the Treasurer.* This report shows an increase in the total assets of the Laboratory from $2,451,630.65 in 1928 to $2,660,478.82 in 1929, the greater part of this increase being due to the addition to the endowment of the Laboratory of a generous gift of $200,000 for the support of the library, from the General Edu-

cation Board. The total income for the year was slightly less than that for 1928, namely, $200,408.91 as compared with $202.825.09; but, as the expenditures decreased to an even greater extent, the excess of expenditures over income, after allowing for depreciation, was $855.33 in 1929, as compared with $2,715.71 in 1928. During the past year the mortgage obligations of the Laboratory were reduced by $4,500, leaving an amount still outstanding of $27,000. The steady progress made during the past five years in the payment of mortgages is indicated by the following figures representing the amounts so owed at the close of each of the years, 1925 to 1929, inclusive:

1925	$54,030.01
1926	44,500.00
1927	42,500.00
1928	31,500.00
1929	27,000.00

3. *The Report of the Librarian.* The past year has shown a continuation of the rapid and substantial gains in the usefulness of the library made possible by the appropriation of $50,000 by the General Education Board in 1926. This appropriation has been paid in unequal annual installments over the past four years as follows: 1926, $10,000; 1927, $15,000; 1928, $15,000; and 1929, $10,000. With the completion of the gift in 1929 it becomes of interest to tabulate the growth of the library during the period in question.

	1925	1926	1927	1928	1929
Serials received currently	500	628	764	874	985
Total number of bound volumes	15,000	18,200	22,800	26,500	28,300
Reprints	25,000	38,000	43,000	51,000	59,000

It will be noted that the numbers of serials received currently and of bound volumes (mostly serials) have almost doubled since 1925, while the number of reprints has considerably more than doubled. So satisfactory has been the progress of the library under the favorable conditions created by the generosity of the General Education Board that the new gift from this body of $200,000, noted above, the income from which will permit a permanent continuation of the remarkable progress of the past four years, is particularly gratifying.

4. *Change in the By-Laws.* At the 1928 meeting of the Board of Trustees the Executive Committee was instructed to make the matter of the constitution of the Board of Trustees a special order of business and after a thorough consideration of the matter it reported as follows at the regular meeting held on August 13, 1929.

"The Board of Trustees is practically a self-perpetuating body comprising men who have been Trustees for many years and whose continued interest and counsel are essential for the continued well-being of the Laboratory. The present policy of re-nominating the eight Trustees whose terms expire each year gives little opportunity, however, for seeding into the Board young men who are to carry on the traditions and management of the Laboratory. Different methods were considered for the relief of this situation and the Executive Committee recommends one such method which is embodied in the amendment to the by-laws as stated below. By this method the number of Trustees is somewhat increased, not, however, by increasing the number in each class, but by creating a new group of counsellors known as Trustees Emeriti. Such Trustees shall have the same rights as other Trustees. Any Trustee upon his seventieth birthday will become automatically a candidate for election by the Corporation at the next annual meeting as Trustee Emeritus for life. By filling the vacancies arising in this way young men can be introduced into the Board gradually, and at the same time we can honor the elder statesmen by electing them to this privileged group.

"We recommend, therefore, the following by-law as a substitute for paragraph I of the present By-laws:

"'The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory in Woods Hole, Massachusetts, at 12 o'clock noon, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years. There shall be thirty-two Trustees thus chosen divided into four classes, each to serve four years and in addition there shall be two groups of Trustees as follows: (a) Trustees *ex officio*, who shall be the President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer and the Clerk; (b) Trustees Emeriti, who shall be elected from the Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next annual meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee Emeritus for life. The Trustees *ex officio* and Emeriti shall each have the same right to vote as the regular Trustees.

"'The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.'"

Upon motion duly made and seconded and after discussion during which it was brought out that similar rights and privileges would be

shared by the existing Trustees Emeriti, it was unanimously voted to approve and adopt as a substitute for Article I of the current By-laws the form as recommended by the Executive Committee.

5. The Board of Trustees. In accordance with the change in the By-laws adopted by the Trustees, it was voted by the Corporation at its regular annual meeting to honor by election as Trustees Emeriti C. R. Crane, H. H. Donaldson, W. B. Scott and E. B. Wilson. At the same meeting H. C. Bradley, R. Chambers, E. N. Harvey, and A. C. Redfield were elected to fill the vacancies thus created, while I. F. Lewis, R. S. Lillie, E. P. Lyon, C. E. McClung, T. H. Morgan, and D. H. Tennent of the Class of 1929 were re-elected as members of the Class of 1933.

6. *The Biological Bulletin.* In the Report of the Director for 1928 the enlargement of the Editorial Board of the Biological Bulletin by the election of six new members was mentioned. During 1929 Dr. A. C. Redfield, Assistant Professor of Physiology in the Harvard Medical School, was chosen as Managing Editor to succeed Dr. C. R. Moore, whose valuable services in this capacity since 1927 will be remembered with gratitude by all persons connected with the Laboratory. Arrangements were made for Dr. Redfield's management of the journal to begin with the first number of Volume LVIII, appearing early in 1930.

7. *International Physiological Congress.* The summer of 1929 was a memorable one because of the visits to the Laboratory for periods ranging from a few hours to several months of most of the foreign physiologists who attended the XIIIth International Physiological Congress held in Boston in August of that year. A part of the entertainment arranged for the foreign members of the Congress was a visit to the Marine Biological Laboratory on Saturday, August 24, immediately after the conclusion of the Boston meetings. With the kind assistance of a number of the summer residents of Woods Hole, the Laboratory was able to act as host on that occasion to between five and six hundred physiologists and their wives. The local Committee, of which W. E. Garrey was Chairman and Selig Hecht Secretary, and of which the other members were: Mrs. J. Malcolm Forbes, E. N. Harvey, F. R. Lillie, M. H. Jacobs, Mrs. E. B. Meigs, A. N. Richards and Mrs. J. P. Warbasse, arranged the following program for the day:

12:30 to 1:30 P.M. Arrival and registration of guests followed by lunch at the Mess.

2:00 to 6:00 P.M. (a) Demonstration of the scientific activities of the Laboratory and of the U. S. Bureau of Fisheries.

(b) Collecting trips on the Cayadetta.
(c) Sailing trips.
(d) Bathing.
(e) Carriage drives on Naushon Island.
(f) Motion pictures of biological interest.
(g) Teas at the M. B. L. Club House, the U. S. Bureau of Fisheries and on Naushon Island.

6:30 P.M. Clam-bake at Gladheim, the residence of Dr. and Mrs. J. P. Warbasse, Penzance Point.

Most of the visitors departed on the night boat for New York; but after a stay of some days in that city, and in some cases after further travel in the United States and Canada, a considerable number returned to Woods Hole, where they were the guests of the Laboratory for further periods ranging from a few days to several weeks. In addition to these visitors, the Laboratory also had the pleasure of entertaining for varying lengths of time before the opening of the Congress a number of specially invited foreign guests whose presence in Woods Hole contributed much to the scientific and the social life of the community.

8. *Lectures.* The year 1929 was also remarkable for both the number and the importance of the evening lectures delivered during the summer. This memorable season was made possible by the kindness of the distinguished foreign visitors who consented to speak on various aspects of their own important work. During the period between June 21 and September 13, sixteen lectures in all were delivered, twelve of them by visiting foreign scientists, representing eight European countries. An examination of the list of lecturers on page 34 will show a collection of distinguished names which it will be very difficult in the future to equal in any single season.

Of the lectures given in 1929, two were special memorial lectures. These were: The Eighth William Thompson Sedgwick Memorial Lecture, delivered on August 16 by Dr. Torsten Thunberg, Professor of Physiology, University of Lund, Sweden, and the First Reynold A. Spaeth Memorial Lecture, delivered on September 9 by Dr. Rudolf Höber, Professor of Physiology, University of Kiel, Germany.

In addition to the more formal lectures already mentioned, there were also held 9 meetings for the presentation and informal discussion of shorter scientific papers. The total number of papers presented at these meetings was 29; their titles are given on page 35. Since meetings of this type appear to fill a real need of the Laboratory, it is planned to continue them in future years.

9. *Courses.* Under the heading of *Attendance* there has already been mentioned the change in the dates between which the various

courses were held in 1929 and the effects of this change in the reduction of crowding. It was anticipated that the new arrangement might result in a considerable temporary decrease in the number of students in attendance, but the actual decrease proved to be much smaller than was anticipated and was in part attributable to factors other than the times at which the courses were held. On the whole, the new plan was so successful that with certain minor modifications which the experience of the past year has shown to be desirable, it will be continued in 1930.

One change in personnel occurred in 1929 in connection with the direction of the courses, Dr. M. H. Jacobs retiring from the position as head of the Physiology Course which he had held since 1922 and Dr. W. R. Amberson being chosen to take his place, beginning in 1930. There should also be mentioned the very successful direction during 1929 of the Embryology Course by Dr. Charles Packard during the temporary absence of its Head, Dr. H. B. Goodrich.

10. *Gifts.* In addition to the important appropriation received from the General Education Board and already mentioned in the discussion of the reports of the Treasurer and of the Librarian, the following additional gifts are gratefully acknowledged: from Dr. Frank R. Lillie, $1,950 for beautifying the grounds of the Laboratory; from Professor Henri Fredericq, $200 for scholarships in Physiology, to be awarded at the discretion of the Executive Committee; and from the "Collecting Net," through Mr. Ware Cattell, $500 for scholarships to be awarded by a committee consisting of Doctors E. G. Conklin, W. E. Garrey and L. L. Woodruff. Further acknowledgment is also made, with the thanks of the Laboratory, of gifts to the library, already noted in the Report of the Librarian, from Dr. H. McE. Knower, Dr. E. Fauré-Fremiet, Mr. Ware Cattell and Dr. H. S. Colton.

There are appended as parts of this report:

1. The Staff, 1929.
2. Investigators and Students, 1929.
3. A Tabular View of Attendance, 1925–1929.
4. Subscribing and Coöperating Institutions, 1929.
5. Evening Lectures, 1929.
6. Shorter Scientific Papers, 1929.
7. Members of the Corporation, August, 1929.

1. THE STAFF, 1929

MERKEL H. JACOBS, *Director*, Professor of General Physiology, University of Pennsylvania.
Associate Director: ——

I. INVESTIGATION

GARY N. CALKINS, Professor of Protozoölogy, Columbia University.
E. G. CONKLIN, Professor of Zoölogy, Princeton University.
CASWELL GRAVE, Professor of Zoölogy, Washington University.
H. S. JENNINGS, Professor of Zoölogy, Johns Hopkins University.
FRANK R. LILLIE, Professor of Embryology, The University of Chicago.
C. E. MCCLUNG, Professor of Zoölogy, University of Pennsylvania.
S. O. MAST, Professor of Zoölogy, Johns Hopkins University.
T. H. MORGAN, Director of the Biological Laboratory, California Institute of Technology.
G. H. PARKER, Professor of Zoölogy, Harvard University.
E. B. WILSON, Professor of Zoölogy, Columbia University.
LORANDE L. WOODRUFF, Professor of Protozoölogy, Yale University.

II. INSTRUCTION

J. A. DAWSON, Instructor in Zoölogy, Harvard University.
RUDOLF BENNITT, Associate Professor of Zoölogy, University of Missouri.
T. H. BISSONNETTE, Professor of Biology, Trinity College.
E. C. COLE, Associate Professor of Biology, Williams College.
MADELEINE P. GRANT, Assistant Professor of Zoölogy, Mount Holyoke College.
E. A. MARTIN, Assistant Professor of Zoölogy, College of the City of New York.
O. E. NELSEN, Instructor in Zoölogy, University of Pennsylvania.
A. E. SEVERINGHAUS, Instructor in Zoölogy, Columbia University.

PROTOZOÖLOGY

I. INVESTIGATION

(*See Zoölogy*)

II. INSTRUCTION

GARY N. CALKINS, Professor of Protozoölogy, Columbia University.
MARY STUART MACDOUGALL, Professor of Zoölogy, Agnes Scott College.
W. B. UNGER, Assistant Professor of Zoölogy, Dartmouth College.

EMBRYOLOGY

I. INVESTIGATION

(*See Zoölogy*)

II. INSTRUCTION

HUBERT B. GOODRICH, Professor of Biology, Wesleyan University.
BENJAMIN H. GRAVE, Professor of Biology, De Pauw University.
LEIGH HOADLEY, Assistant Professor of Zoölogy, Harvard University.

CHARLES PACKARD, Assistant Professor of Zoölogy, Institute of Cancer Research, Columbia University.
HAROLD H. PLOUGH, Professor of Biology, Amherst College.
CHARLES G. ROGERS, Professor of Comparative Physiology, Oberlin College.

PHYSIOLOGY

I. INVESTIGATION

HAROLD C. BRADLEY, Professor of Physiological Chemistry, University of Wisconsin.
WALTER E. GARREY, Professor of Physiology, Vanderbilt University Medical School.
RALPH S. LILLIE, Professor of General Physiology, The University of Chicago.
ALBERT P. MATHEWS, Professor of Biochemistry, The University of Cincinnati.

II. INSTRUCTION

MERKEL H. JACOBS, Professor of General Physiology, University of Pennsylvania.
WILLIAM R. AMBERSON, Assistant Professor of Physiology, University of Pennsylvania.
EDWIN J. COHN, Assistant Professor of Physical Chemistry, Harvard University.
E. NEWTON HARVEY, Professor of Physiology, Princeton University.
CHARLOTTE HAYWOOD, Assistant Professor of Physiology, Vassar College.
SELIG HECHT, Associate Professor of Biophysics, Columbia University.
LEONOR MICHAELIS, Resident Lecturer in Medical Research, Johns Hopkins University.
ALFRED C. REDFIELD, Assistant Professor of Physiology, Harvard University.

BOTANY

I. INVESTIGATION

B. M. DUGGAR, Professor of Physiological and Economic Botany, University of Wisconsin.
C. E. ALLEN, Professor of Botany, University of Wisconsin.
S. C. BROOKS, Professor of Zoölogy, University of California.
IVEY F. LEWIS, Professor of Biology, University of Virginia.
WM. J. ROBBINS, Professor of Botany, University of Missouri.

II. INSTRUCTION

WILLIAM RANDOLPH TAYLOR, Professor of Botany, University of Pennsylvania.
JAMES P. POOLE, Professor of Evolution, Dartmouth College.
ETHEL M. POULTON, Leessel Fellow, Yale University.

LIBRARY

PRISCILLA B. MONTGOMERY (Mrs. Thomas H. Montgomery, Jr.), Librarian.
DEBORAH LAWRENCE, Secretary.
HESTER ANN BRADBURY, LILLIAN F. BRIGGS, MARY A. ROHAN, Assistants.

CHEMICAL SUPPLIES

OLIVER S. STRONG, Professor of Neurology, and Neuro-Histology, Columbia University, *Chemist.*

APPARATUS ROOM

SAMUEL E. POND, Assistant Professor of Physiology, Medical School, University of Pennsylvania, *Custodian of Apparatus.*

SUPPLY DEPARTMENT

GEORGE M. GRAY, Curator.
JOHN J. VEEDER, Captain.
E. M. LEWIS, Engineer.
A. W. LEATHERS, Head of Shipping Department.
A. M. HILTON, Collector.
J. MCINNIS, Collector.
MILTON B. GRAY, Collector.

F. M. MACNAUGHT, Business Manager.
HERBERT A. HILTON, Superintendent of Buildings and Grounds.
THOMAS LARKIN, Superintendent of Mechanical Department.
LESTER F. BOSS, Mechanician.
WILLIAM HEMENWAY, Carpenter.
J. D. GRAHAM, Glass-blowing Service.
A. R. APGAR, Photographic Service.
ARNOLD H. BISCO, Storekeeper and Head Janitor.

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2. INVESTIGATORS AND STUDENTS, 1929

Independent Investigators

ADDISON, WILLIAM H. F., Professor of Normal Histology and Embryology, University of Pennsylvania.
ALLEN, C. E., Chairman, Division of Biology and Agriculture, National Research Council.
AMBERSON, WILLIAM R., Assistant Professor of Physiology, University of Pennsylvania.
ARMSTRONG, PHILIP, Instructor in Anatomy, Cornell University Medical College.
ASHER, LEON, Director, Physiological Institution, Berne, Switzerland.
ASHER, MRS. LEON, Physiological Institution, Berne, Switzerland.
AUSTIN, MARY L., Instructor in Zoölogy, Wellesley College.
BARCROFT, JOSEPH, Professor of Physiology, Cambridge University, England.
BARRON, E. S. GUZMAN, Instructor in Medicine, Johns Hopkins University.
BARTH, L. G., Graduate Student, University of Chicago.
BELAR, K. J., Kaiser Wilhelm Institut für Biologie, Berlin-Dahlem, Germany.
BENNITT, RUDOLF, Associate Professor of Zoölogy, University of Missouri.
BETHE, A., Professor, Institut f. Animalische Physiologie, Frankfurt, Germany.
BIGELOW, ROBERT P., Professor of Zoölogy, Massachusetts Institute of Technology.
BISSONNETTE, T. HUME, Professor of Biology, Trinity College.
BLAKE, CHARLES H., Instructor, Massachusetts Institute of Technology.
BLANCHARD, KENNETH C., Assistant Professor of Biochemistry, Washington Square College, New York University.
BLUMENTHAL, REUBEN, Graduate Student, University of Pennsylvania.

BORODIN, DANIEL, Pasadena, California.
BOWEN, ROBERT H., Professor of Zoölogy, Columbia University.
BOYD, GEORGE H., Professor of Zoölogy, University of Georgia.
BRADLEY, HAROLD C., Professor of Physiological Chemistry, University of Wisconsin.
BREBNER, WILLIAM B., Washington University Medical School.
BREITENBECHER, J. K., Research Assistant and Lecturer in Zoölogy, McGill University.
BRIDGES, CALVIN B., Research Assistant, Carnegie Institution of Washington.
BRINLEY, FLOYD J., Professor of Physiology, Battle Creek College.
BROOKS, MATILDA M., Research Associate in Biology, University of California.
BUDINGTON, ROBERT A., Professor of Zoölogy, Oberlin College.
BUISSET, SIMONE, C. R. B. Fellow, University of Pennsylvania.
BUTCHER, EARL, Assistant Professor, Hamilton College.
BUTLER, ELMER G., Assistant Professor of Biology, Princeton University.
CALKINS, GARY N., Executive Officer, Department of Zoölogy, Columbia University.
CANNAN, ROBERT K., Senior Lecturer, University College, London, England.
CARMICHAEL, EMMETT B., Associate Professor and Head of Department of Physiological Chemistry, School of Medicine, University of Alabama.
CAROTHERS, E. ELEANOR, Lecturer in Zoölogy, University of Pennsylvania.
CARROLL, PAUL L., Instructor in Biology, Saint Louis University.
CARVER, G. L., Professor of Biology, Mercer University.
CATTELL, MCKEEN, Assistant Professor of Physiology, Cornell University Medical College.
CATTELL, WARE, Research Fellow in Biophysics, Memorial Hospital.
CHAMBERS, ROBERT, Research Professor of Biology and Chairman of Department of Biology, Washington Square College, New York University.
CHEMIN, EMILE A., Professor, Lycée, Buffon, Paris, France.
CHENEY, RALPH H., Assistant Professor of Biology, Washington Square College, New York University.
CHIDESTER, F. E., Professor of Zoölogy, West Virginia University.
CHOUKE, K. S., Instructor in Anatomy, University of Colorado, School of Medicine.
CHRISTIE, JESSE R., Associate Nematologist, United States Department of Agriculture.
CLARK, ELEANOR L., Investigator, University of Pennsylvania.
CLARK, ELIOT R., Professor of Anatomy, University of Pennsylvania.
CLOWES, G. H. A., Director, The Lilly Research Laboratory, Eli Lilly & Co.
COBB, N. A., Nematologist, United States Department of Agriculture.
COE, WESLEY R., Professor of Biology, Yale University.
COHEN, BARNETT, Associate Professor of Physiological Chemistry, Johns Hopkins Medical School.
COLE, ELBERT C., Associate Professor of Biology, Williams College.
CONKLIN, EDWIN G., Research Professor of Biology, Princeton University.
COPELAND, MANTON, Professor of Biology, Bowdoin College.
COWDRY, E. V., Professor of Cytology, Washington University Medical School.
COWLES, R. P., Associate Professor of Zoölogy, Johns Hopkins University.
CRABB, E. D., Instructor in Zoölogy, University of Pennsylvania.
CURTIS, W. C., Professor of Zoölogy, University of Missouri.
DAVIS, EVERETT F., Assistant Plant Physiologist, Virginia Agricultural Experiment Station.
DAVIS, FANNY-FERN SMITH, Assistant Plant Physiologist, Virginia Agricultural Experiment Station.
DAVIS, HALLOWELL, Assistant Professor of Physiology, Harvard University Medical School.

DAWSON, J. A., Instructor in Invertebrate Zoölogy, Harvard University.
DECARO, LUIGI, Assistant, University of Naples, Naples, Italy.
DOBZHANSKY, THEODOSIUS, Research Fellow, International Education Board.
DODDS, G. S., Professor of Histology and Embryology, West Virginia University Medical School.
DOLLEY, WM. L., Professor of Biology, University of Buffalo.
DONALDSON, HENRY H., Member, Wistar Institute.
DONALDSON, JOHN C., Associate Professor of Anatomy, University of Pittsburgh.
DU BOIS, EUGENE F., Associate Professor of Medicine, Cornell University Medical College.
EDWARDS, DAYTON J., Associate Professor of Physiology, Cornell University Medical College.
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Finkelstein, Ruth, Johns Hopkins University.
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3. TABULAR VIEW OF ATTENDANCE

	1925	1926	1927	1928	1929
INVESTIGATORS—Total	207	252	294	323	329
Independent	135	156	209	217	234
Under Instruction	72	84	57	81	71
Research Assistants		12	28	25	24
STUDENTS—Total	132	141	141	133	125
Zoölogy	54	56	57	57	53
Protozoölogy	17	19	17	16	15
Embryology	29	28	32	29	28
Physiology	19	18	19	15	17
Botany	13	20	16	16	12
TOTAL ATTENDANCE	339	393	435	456	454
Less Persons registered as both students and investigators		8	1	2	10
		385	434	454	444
INSTITUTIONS REPRESENTED—Total	112	119	111	111	123
By investigators	74	84	89	80	96
By students	65	60	63	66	64
SCHOOLS AND ACADEMIES REPRESENTED					
By investigators	1	–	1	1	
By students	4	4	4	1	1
FOREIGN INSTITUTIONS REPRESENTED					
By investigators	–	17	15	13	30
By students	–	3	8	8	3

4. SUBSCRIBING AND COOPERATING INSTITUTIONS

Acadia University
Agnes Scott College
Amherst College
Barnard College
Battle Creek College
Bowdoin College
Brown University
Bryn Mawr College
Butler College
C. R. B. Educational Foundation
California Institute of Technology
Carnegie Institution, Cold Spring Harbor
Carnegie Institution of Washington
Chinese Educational Mission
Columbia University
Commonwealth Fund
Cornell University
Cornell University Medical College
De Pauw University
Duke University
Elmira College
General Education Board
Goucher College
Hamilton College
Harvard University
Harvard University Medical School
Howard University
Industrial & Engineering Chemistry, of the American Chemical Society
International Education Board
Johns Hopkins University
Johns Hopkins University Medical School
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United States Dept. of Agriculture
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Ida H. Hyde Scholarship of the University of Kansas.
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Scholarship of $100 supported by a friend of the Laboratory since 1898.
The Edwin S. Linton Memorial Scholarship of Washington and Jefferson College.
The Bio Club Scholarship of the College of the City of New York.

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5. EVENING LECTURES, 1929

Friday, June 21
DR. SELIG HECHT ..... "The Nature of Visual Acuity."
Friday, June 28
DR. PAUL S. GALTSOFF ..... "The Physiology of Feeding and Reproduction of the American Oyster."
Friday, July 5
DR. E. M. EAST ..... "Immunological Phenomena in Plants."

Friday, July 12
Dr. G. H. Parker ..... "The Nature of Neurofibrils."
Friday, July 19
Dr. E. Fauré-Fremiet ..... "La Structure des amoebocytes hyalins et leurs propriétés physiologiques."
Friday, July 26
Dr. Henri Fredericq ..... "Humoral Transmission of Nerve Action."
Friday, August 2
Dr. P. B. Rehberg ..... "Studies on Kidney Function."
Friday, August 9
Dr. Hans Winterstein ..... "The Metabolism of the Process of Excitation."
Friday, August 16
The William Thompson Sedgwick Memorial Lecture, delivered by Dr. Torsten Thunberg ..... "The Hydrogen-Activating Enzymes of the Cells."
Friday, August 23
Dr. K. T. Belar ..... "Investigations on the Structure and Function of the Mitotic Spindle."
Friday, August 30
Dr. H. H. Dale ..... "Some Chemical Factors in the Regulation of the Blood Flow."
Tuesday, September 3
Dr. August Krogh ..... "Respiratory Mechanisms in Insects."
Friday, September 6
Dr. Joseph Barcroft ..... "Recent Observations on the Spleen."
Monday, September 9
The Reynold A. Spaeth Memorial Lecture, delivered by Dr. Rudolf Höber ..... "The Structure of the Plasma Membrane."
Wednesday, September 11
Dr. Frederic Verzar ..... "On the Mechanism of Absorption from the Intestine."
Friday, September 13
Dr. Enrico Sereni ..... "The Physiology of Chromatophores."

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6. SHORTER SCIENTIFIC PAPERS, 1929

Tuesday, July 2
Dr. J. M. Johlin ..... "The Surface Tension of Solutions of Semicolloids in Relation to the Concentration."
Dr. L. Michaelis ..... "The Mechanism of the Iron Catalysis in Oxidation."

Tuesday, July 9

Dr. Abby H. Turner ..... "The Adjustment of the Human Circulation in Prolonged Standing."

Dr. W. E. Garrey ..... "The Non-existence of Physiological Leucocytosis."

Dr. D. J. Edwards and
Dr. J. McKeen Cattell..... "Some Physiological Effects of Hydrostatic Pressure."

Tuesday, July 16

Mr. L. G. Barth ..... "The Effects of Acids and Alkalies on the Viscosity of Arbacia Egg Protoplasm."

Dr. L. V. Heilbrunn ..... (a) "The Viscosity of Amoeba Protoplasm at Different Temperatures." (b) "Further Study of the Surface Precipitation Reaction."

Dr. Paul Reznikoff ..... "Response of White Blood Cells to Stimulation."

Tuesday, July 23

Mr. Harry Grundfest ..... "Visibility of the Spectrum to the Fish Eye."

Dr. Wm. L. Dolley, Jr. ..... "Some Visual Phenomena in Eristalis tenax."

Dr. H. K. Hartline ..... "The Course of Dark Adaptation of the Eye of Limulus as Manifested by its Electrical Response."

Tuesday, July 30

Mr. Reuben Blumenthal ..... "Some Effects of Cyanides in Relation to Mitosis in Arbacia Eggs."

Dr. Henry J. Fry ..... "A Critique of the Usual Concepts Concerning the Mitotic Mechanism of the Echinoderm Egg."

Dr. Boris Sokoloff ..... "Liquefaction of Malignant Tumors and Iron Metabolism."

Tuesday, August 6

Dr. A. Franklin Shull ..... "Intermediate Aphids and Goldschmidt's Theory of Intersexes."

Dr. C. W. Metz ..... "Unisexual Progenies and the Mechanism of Sex-determination in Sciara."

Dr. T. Dobzhansky ..... "Chromosome Translocation in Drosophila Induced by X-rays."

Tuesday, August 13

Miss Helen B. Smith ..... "Depression in Rotifera."

Mr. Oscar W. Richards ..... "The Later Part of the Growth of a Population of Yeast."

Dr. Maynard M. Metcalf ..... "An African Zoögeographical Puzzle."

Tuesday, August 20

Dr. Mary S. MacDougall	"Modifications in Chilodon unicinatus Produced by Ultra-violet Radiation."
Miss Rachael Bowling	"Conjugation of Distomatous Individuals of Glaucoma Scintillans."
Dr. Gary N. Calkins	"Chromosomes in Uroleptus Halseyi."
Dr. J. A. Dawson	"Motion Pictures of Living Protozoa."

Tuesday, August 27

Dr. J. Nitzulescu, Mr. T. Kerr and Mr. G. A. Millikan	"Salt Errors of Clark-Lubs Indicators for Woods Hole Sea Water."
Dr. Sergius Morgulis	"Blood Calcium in Tetany."
Dr. S. E. Pond	"Elementary Study of the Bone Solids with Particular Reference to Age."
Dr. E. S. Guzman Barron and Dr. L. B. Flexner	"The Reduction Potential of Sugars."
Dr. Kenneth Blanchard	(a) "Inorganic Constituents of the Arbacia Egg." (b) "Autoxidation of Unsaturated Hydrocarbons."

7. MEMBERS OF THE CORPORATION

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THE INFLUENCE OF CARBON DIOXIDE UPON THE OXYGEN CONSUMPTION OF PARAMECIUM AND THE EGG OF ARBACIA

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The inter-relationship between oxygen and carbon dioxide in supporting the respiration of higher animals is a matter of fundamental importance. Since experiments upon such forms are often difficult to interpret, because of the number of uncontrollable factors involved, single cells have been used in the present study in which the material employed was *Paramecium caudatum* and the fertilized eggs of *Arbacia punctulata*.

Few investigations have been made concerning the effect of carbon dioxide upon the oxygen consumption of protozoa or of marine eggs, though Warburg (1910) observed no change in the respiration of fertilized sea urchin eggs in the presence of a carbon dioxide tension of 15 mm. Hg, and Burfield (1928) reported that small amounts of carbon dioxide profoundly decreased the rate of oxygen consumption of plaice eggs. In these studies, however, oxygen was measured by the Winkler method which, while accurate for the determination of dissolved oxygen in solutions free of organic materials, is said to be untrustworthy for egg suspensions (Heilbrunn, 1915; Warburg, 1914*a*). Moreover, the presence of iron in sea urchin eggs (Warburg, 1914*b*) is known to introduce large errors into the method (Alsterberg, 1926). Previous work on the effect of carbon dioxide upon the rate of oxygen consumption of single cells appears, therefore, to be not entirely satisfactory and a reinvestigation of the question by other methods has seemed desirable. In the experiments here recorded, the technique employed was that developed by Novy and his collaborators (1925) for bacterial respiration, and modified by Amberson (1928) for the respiration of unicellular animal organisms.

Experiments with Paramecium

A thick suspension of the protozoa was obtained by placing several liters of culture in a large glass cylinder which was illuminated near

the top by rays of light from a sixty-watt electric light bulb. The organisms under these conditions swim to the top, and may be siphoned off in great numbers. The suspension so obtained was then centrifuged at two thousand revolutions a minute for ten to twenty seconds. The centrifuge was stopped suddenly to prevent the organisms from swimming from the bottom of the centrifuge tubes before the supernatant fluid could be decanted. The sediment of protozoa was washed in several changes of boiled, cooled, filtered pond water. The suspension in its final form must have been relatively free from bacteria, because control experiments with the cells absent showed no measurable oxygen consumption. The cultures were never pure, but *P. caudatum* always constituted at least 95 per cent of the protozoa present. The original culture of *Paramecium* was obtained from Dr. William Canovan of the Zoölogy Department of the University of Pennsylvania.

In order to obtain two suspensions containing approximately the same number of cells, a calibrated glass "mixer" was used. This consisted of a glass tube three-quarters of an inch in diameter, fitted at each end with a ground glass stopper. It was separated into two chambers of about twenty cc. capacity by a stopcock, the bore of which was of the same diameter as the tube. The suspension was poured into and out of the "mixer" eight to ten times with one stopper in place and with the stopcock open. After pouring the suspension in for the last time, the second stopper was inserted and the stopcock turned before the protozoa had an opportunity to change their distribution. The suspensions on each side of the stopcock were then poured into two calibrated cylindrical glass vessels of about the size and shape of Haldane gas collecting tubes. These tonometers were fitted with three-way stopcocks at both ends. Twenty-five cc. of boiled, cooled, filtered pond water were added to each tonometer.

Gas mixtures were made up in two twenty-one liter bottles so arranged that, when air or nitrogen was forced into one, the water contained therein passed into the second bottle, displacing the gas mixture which had previously been made up to approximately the desired percentage of oxygen and carbon dioxide. While the water contained in these bottles absorbed a certain amount of the gas mixtures above it, the gas tensions were determined from samples of the gas after it had passed through the tonometer, and immediately before the tonometer was closed. Any changes in gas tension occurring within the bottles did not, therefore, result in errors in the respiratory determinations.

At the beginning of an experiment, the gas mixture was slowly bubbled through one of the suspensions. Temperature equilibrium was

achieved by placing the tonometers in a water bath at the same temperature as that at which the respiration was to be measured (25.6° ± 0.3° C.). Every few seconds the passage of the gas was suspended and, with the stopcocks closed, the tonometer was gently rocked to hasten the attainment of equilibrium. After such a procedure, a drop of water was left in the capillary part of the tonometer which leads to the stopcock. When the tonometer was opened to the outside air, the drop was displaced inwards, because the pressure within the tonometer was less than atmospheric, due to the absorption of gases by the suspension. Passage of the gas mixture was continued until the drop was no longer displaced. A sample of the gas mixture, after it had passed through the tonometer, was now collected in a Bailey sampling bottle. Care was exercised to prevent the presence of a positive pressure within the tonometer. The stopcocks were then closed and the tonometers were placed in a second water bath. The tonometers were rotated upon their long axes sixty times a minute. No cellular destruction or abnormal behavior was observed. The same cultures were used every second or third day.

While the first suspension was undergoing the above treatment, the second suspension was equilibrated in the same water bath with air, by connecting one end of the tonometer with a compressed air inlet or a water pump. After approximately the same length of time as was required for the equilibration of the tonometer containing carbon dioxide, an air sample was secured, the stopcocks were closed, and the tonometer was placed in the rotator. The two tonometers rarely differed by more than five to ten minutes in their starting times. At the end of two to four hours, the tonometers were removed from the water bath and a sample of the contained gas was withdrawn in a Bailey collector, and set aside for later analysis. Respiratory exchanges were calculated for three-hour experiments.

The gas samples were analyzed in duplicate by means of a Haldane-Henderson analyzer with a nitrogen side tube, the principle being that employed in Bazett's modification (1928). The analyses were controlled by daily air analyses, and were accurate to 0.03 to 0.04 per cent.

Assuming the gaseous solubilities to be those given by the standard tables for pure water at this temperature, the total oxygen and carbon dioxide present at the beginning and at the end of the experiment in both air and water were calculated, the usual correction for barometer, water vapor, etc., being applied. The results of a typical experiment are as follows:

SAMPLE EXPERIMENT

Tonometers	VI	V
Volume of tonometers	99.36 cc.	99.47 cc.
Volume of suspension	45.01 cc.	45.01 cc.
Gas analysis at beginning		
O_2	20.91%	19.29%
CO_2	0.04%	7.50%
N_2	79.05%	73.21%
Gas analysis at end (corrected for volume change)		
O_2	18.71%	17.22%
CO_2	0.88%	8.61%
N_2	79.05%	73.21%
Oxygen in air and water		
at beginning	11.626 cc.	10.746 cc.
at end	10.403 cc.	9.593 cc.
Oxygen consumption	1.223 cc.	1.153 cc.
Carbon dioxide in air and water		
at beginning	0.036 cc.	6.788 cc.
at end	0.793 cc.	7.789 cc.
Carbon dioxide production	0.757 cc.	1.001 cc.
Volumes corrected to dry values at 0° C. and 760 mm. Hg		
Oxygen consumption	1.081 cc.	1.019 cc.
Carbon dioxide production	0.669 cc.	0.885 cc.
Respiratory quotient	0.618	0.868
Oxygen tension in mm. Hg	153.4–137.2	141.5–126.3
Carbon dioxide tension in mm. Hg	0.3– 6.4	55.0–63.1

Ratio oxygen consumption $\frac{V}{VI} = 0.942$

Ratio carbon dioxide production $\frac{V}{VI} = 1.322$

The results obtained in forty-five experiments are given in Table I and Fig. 1. It will be noted that at a carbon dioxide tension of about 15 mm. Hg, the curve of the rate of oxygen consumption rises, reaching a maximum at approximately 40 mm. Hg and, crossing the line of the control rate of oxygen consumption at about 67 mm. Hg, falls away at the higher tensions.

Examination of the tonometers with a low powered binocular microscope showed that an exposure of three hours to a carbon dioxide tension of about 150 mm. Hg noticeably decreased the motility of the protozoa. At about 220 mm. Hg the cells became shorter and thicker and the nuclei became more clearly visible, standing out sharply from the rest of the protoplasm. When the animals were subjected to a tension of 250 mm. Hg for three hours, some were irreversibly affected by the gas.

In all experiments, the oxygen tension was kept above 62 mm. Hg. Amberson (1928) has shown that the rate of oxygen consumption of *Paramecium* is constant between 200 and 50 mm. Hg partial pressure, so it is unlikely that the results here reported were due to a lack of

TABLE I

Respiration of Paramecium at Different Carbon Dioxide Tensions

Experiment No.	CO_2 Tensions in Tonometer B	Respiration in Tonometer A			Respiration in Tonometer B			Ratio O_2 Consumption $\frac{B}{A}$	Ratio CO_2 Production $\frac{B}{A}$
		O_2 Consumed	CO_2 Produced	RQ	O_2 Consumed	CO_2 Produced	RQ		
	mm. Hg	*cc.*	*cc.*		*cc.*	*cc.*			
1	0.4– 3.6	0.381	0.256	0.669	0.357	0.254	0.711	0.936	0.999
2	0.3– 12.1	1.159	0.871	0.751	1.116	0.924	0.827	0.962	1.060
3	0.4– 7.4	1.169	0.731	0.625	1.154	0.692	0.599	0.987	0.946
4	1.0– 8.1	1.264	0.647	0.511	1.302	0.732	0.562	0.970	1.131
5	16.7– 26.4	1.305	0.848	0.650	1.315	1.042	0.780	1.007	1.228
6	25.8– 33.9	0.776	0.535	0.691	0.817	0.895	1.096	1.052	1.672
7	29.1– 39.1	1.232	0.813	0.659	1.359	1.083	0.796	1.103	1.332
8	34.4– 37.4	0.410	0.288	0.702	0.452	0.347	0.729	1.102	1.204
9	39.3– 43.1	0.592	0.266	0.450	0.652	0.315	0.484	1.101	1.184
10	42.7– 50.3	0.813	0.474	0.613	0.907	0.713	0.823	1.115	1.504
11	43.0– 52.3	1.565	0.810	0.525	1.628	1.004	0.617	1.040	1.239
12	44.8– 54.2	0.813	0.511	0.617	0.886	1.012	1.144	1.067	1.980
13	48.0– 49.8	0.572	0.296	0.519	0.574	0.181	0.318	1.004	0.611
14	50.9– 58.2	1.314	0.789	0.601	1.485	0.779	0.525	1.130	0.987
15	52.4– 61.4	1.235	0.776	0.619	1.312	0.977	0.740	1.047	1.259
16	62.5– 69.5	1.117	0.599	0.536	1.187	0.763	0.642	1.062	1.273
17	69.3– 75.3	1.622	0.923	0.569	1.504	0.651	0.432	0.927	0.705
18	69.8– 79.0	1.674	0.925	0.552	1.573	0.889	0.565	0.939	0.961
19	80.5– 90.9	2.370	1.119	0.472	2.118	1.142	0.539	0.893	1.020
20	90.6– 97.3	0.774	0.554	0.716	0.644	0.662	1.027	0.832	1.176
21	91.4–110.8	3.260	1.859	0.570	3.021	2.018	0.668	0.926	1.085
22	94.7–100.9	0.864	0.643	0.744	0.810	0.589	0.727	0.937	0.916
23	102.6–118.8	2.417	1.448	0.599	2.222	1.689	0.760	0.919	1.166
24	105.6–127.4	3.839	2.152	0.565	3.495	2.334	0.667	0.910	1.089
25	126.7–135.3	0.863	0.565	0.654	0.736	0.814	1.105	0.852	1.440
26	140.9–149.9	1.197	0.812	0.678	0.950	0.964	1.014	0.793	1.187
27	141.6–151.4	0.872	0.645	0.739	0.594	1.071	1.803	0.681	1.660
28	155.0–164.8	1.228	0.789	0.642	0.927	1.004	1.083	0.754	1.272
29	158.2–166.2	1.710	1.015	0.593	1.333	1.213	0.909	0.779	1.195
30	160.8–171.2	1.246	0.888	0.712	0.791	1.027	1.298	0.634	1.156
31	167.3–173.7	0.827	0.514	0.621	0.534	0.473	0.885	0.645	0.920
32	173.8–185.3	2.196	1.308	0.595	1.494	1.334	0.893	0.680	1.019
33	181.8–192.8	1.293	0.997	0.771	0.892	0.867	0.972	0.689	0.869
34	202.2–204.1	0.970	0.667	0.687	0.506	0.184	0.363	0.521	0.275
35	206.8–210.8	1.499	0.946	0.631	0.702	0.395	0.562	0.469	0.418
36	217.4–222.0	2.032	1.124	0.553	1.004	0.525	0.521	0.494	0.467
37	224.7–229.4	0.828	0.488	0.589	0.511	0.502	0.982	0.617	1.028
38	224.9–233.5	0.918	0.509	0.554	0.317	0.914	2.812	0.345	1.795
39	234.5–241.2	0.610	0.385	0.631	0.360	0.713	1.980	0.590	1.851
40	238.2–246.8	0.990	0.673	0.679	0.504	0.713	1.414	0.509	1.059
41	253.3–257.1	1.296	0.679	0.524	0.556	0.385	0.692	0.429	0.567
42	270.2–276.9	1.177	0.787	0.629	0.454	0.693	1.526	0.385	0.880
43	277.2–296.7	1.606	1.010	0.628	0.852	1.711	2.008	0.530	1.694
44	353.9–361.0	1.558	1.017	0.652	0.843	0.715	0.848	0.541	0.703
45	418.1–423.8	0.860	0.398	0.462	0.288	0.599	2.079	0.334	1.505

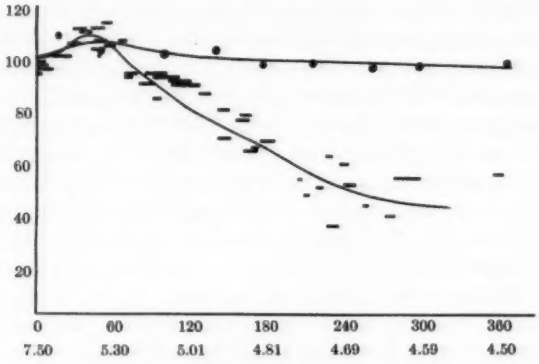

FIG. 1. The effect of carbon dioxide upon the oxygen consumption of *Paramecium*.

Abscissa: upper row of figures = mm. Hg carbon dioxide tension, lower row of figures = pH.

Ordinate = $\frac{\text{oxygen consumption in carbon dioxide tonometer}}{\text{oxygen consumption in control or air tonometer}}$.

— = carbon dioxide experiments.

● = hydrochloric acid experiments.

oxygen. It is, of course, possible that the oxygen tension at which the effects of oxygen deficiency appear, may not be the same in the presence of high tensions of carbon dioxide as in the relative absence of this gas. If the above experiments had been carried out in the region of oxygen deficiency, however, even slight increases in the oxygen tension would have resulted in an increased rate of oxygen consumption. That this was not the case is shown by a comparison of experiments 18 and 19 (Table II) which show that when the oxygen tension in tonometer *B* (CO_2) in experiment 18 was 62 per cent greater than that in tonometer *B* (CO_2), experiment 19, the differences in the rates of oxygen consumption lay within the error of the method. The carbon dioxide tensions in the two tonometers B (CO_2) do not differ by enough to affect appreciably the rates of the oxygen consumption in the two experiments.

TABLE II

Experiment No.	CO_2 tension	Tonometer A O_2 tension	O_2 Consumed	CO_2 tension	Tonometer B O_2 tension	O_2 Consumed	Ratio O_2 Consumption B/A
	mm. Hg	*mm. Hg*		*mm. Hg*	*mm. Hg*		
18	0.30– 9.20	156.5–130.3	1.758	69.8–79.0	142.27–115.8	1.504	0.939
19	0.30–10.64	156.8–121.4	2.370	80.5–90.9	93.8 – 62.4	1.774	0.893

The average of the respiratory quotients obtained in air in forty-eight determinations was 0.62. This is somewhat lower than the average value 0.69 obtained by Amberson (1928) in fourteen experiments. An examination of the respiratory quotients in the presence of different carbon dioxide tensions (Table I) shows that these values fluctuate irregularly between 0.32 and 2.81. When the forty-two experiments, carried out at different carbon dioxide tensions, and their controls are arranged in seven groups of six experiments each, and the respiratory quotients, and ratios of oxygen consumption and carbon dioxide production averaged, Table III is obtained. It will be seen that the rate of carbon dioxide production in the presence of carbon dioxide does not decrease as does the rate of oxygen consumption, but remains relatively constant. As a consequence, the respiratory quotients rise progressively as the carbon dioxide tension is increased.

TABLE III

Summary of Experiments in Table I (Paramecium caudatum)

Group	Experiments	CO_2 tension mm. Hg	*RQ* control	*RQ* CO_2 tonometer	Ratio O_2 Consumption $\frac{CO_2 \text{ tonometer}}{\text{control}}$	Ratio CO_2 Production $\frac{CO_2 \text{ tonometer}}{\text{control}}$
I.....	4–9	1.0– 43.1	0.610	0.741	1.056	1.289
II....	10–15	42.7– 61.4	0.582	0.694	1.067	1.096
III...	16–21	62.0–110.8	0.569	0.645	0.929	1.036
IV...	22–27	91.4–151.4	0.663	1.012	0.848	1.242
V....	28–33	155.0–192.0	0.655	1.006	0.696	1.072
VI...	34–39	202.0–246.0	0.574	1.205	0.506	0.972
VII..	40–45	238.0–423.0	0.595	1.428	0.454	1.068

It has been clearly shown that carbon dioxide enters cells with ease (Jacobs 1920*a*, 1920*b*, 1924), whereas hydrochloric acid penetrates cells very slowly, if at all (Loeb, 1909, Jacobs 1924). In order to determine whether carbon dioxide decreases the rate of oxygen consumption by some internal action on the cell, or merely by increasing the acidity of the external solution, a group of nine experiments was performed to determine the effects upon the respiration of *Paramecium* of approximately the same degrees of acidity produced by hydrochloric acid.

Boiled, filtered, cooled, pond water was equilibrated at different carbon dioxide tensions, and the hydrogen ion concentrations determined electrometrically, using a closed quinhydrone electrode (Fig. 2). The pond water, though poorly buffered, contained bicarbonate in a concentration approximately 0.00014 M. This was sufficient to give relatively stable potentials. The pH readings were accurate to 0.04 of a pH unit as shown by determinations made upon known solutions and by comparing a series of readings.

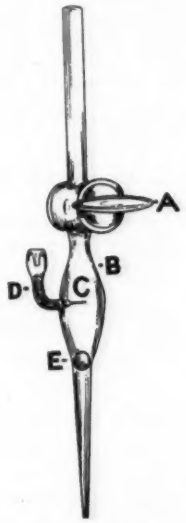

FIG. 2. Closed quinhydrone electrode.

To obtain hydrogen ion concentrations by means of hydrochloric acid, corresponding to those produced by carbon dioxide, pond water was made bicarbonate free by adding concentrated hydrochloric acid to bring it to a pH of 3 to 4, and aerated over night. It was then returned to the desired pH by the addition of concentrated sodium hydroxide.

The calculations of oxygen consumption and carbon dioxide production at different pH values appear in Table IV and Fig. 1. These results indicate that hydrochloric acid has no effect upon the respiration of *P. caudatum* at pH values as low as 4.5. The protozoa appeared in no way injured by these experiments.

TABLE IV

The Effect of Hydrochloric Acid on the Respiration of Paramecium

Experiment No.	Tonometer B pH	Resp. in Tonometer A			Resp. in Tonometer B			Ratio O_2 Consumption $\frac{B}{A}$
		O_2 Consumed	CO_2 Produced	*RQ*	O_2 Consumed	CO_2 Produced	*RQ*	
		cc.	*cc.*		*cc.*	*cc.*		
1	5.85	0.752	0.626	0.832	0.813	0.606	0.745	1.081
2	5.40	0.863	0.526	0.609	0.887	0.549	0.618	1.026
3	5.07	1.113	0.673	0.604	1.119	0.628	0.561	1.005
4	4.90	0.970	0.473	0.487	0.992	0.513	0.517	1.022
5	4.80	1.202	0.830	0.690	1.157	0.875	0.756	0.962
6	4.71	0.805	0.614	0.762	0.783	0.617	0.786	0.972
7	4.61	1.229	0.868	0.706	1.178	0.928	0.787	0.958
8	4.55	0.977	0.561	0.574	0.942	0.659	0.699	0.964
9	4.50	0.618	0.466	0.754	0.610	0.540	0.885	0.987

EXPERIMENTS ON FERTILIZED ARBACIA EGGS

Since it was impossible to determine how much of the depression in the rate of oxygen consumption of *Paramecium* was due to a suppression of the oxidative mechanism, and how much was dependent upon the decreased motility of the organism, it was necessary to perform similar experiments upon a non-motile cell. Experiments were, therefore, carried out on the fertilized eggs of *Arbacia punctulata* at Woods Hole.

The eggs were removed from the female, freed of ovarian debris by straining through cheese cloth, and washed in several changes of sea water. A heavy suspension of cells was secured by allowing the eggs to sediment in several finger bowls, and decanting the supernatant sea water. Eggs from twelve to fifteen females were used in each experiment. After fertilization, the excess spermatozoa were removed by allowing the cells to sediment in several changes of sea water. The suspension was then divided into two parts by means of the "mixer" used in the experiments with *Paramecium*. The two suspensions were equal to within 10 per cent as measured by the respiratory exchange in control experiments. The tonometers used in the experiments on *Paramecium* were also employed in these experiments.

At times varying from twenty to sixty minutes after fertilization, the suspensions were equilibrated simultaneously with air and with different carbon dioxide tensions in the same manner as described for *Paramecium*. At the conclusion of the equilibration in the water bath, the two tonometers were closed in such a manner that the contained gas was left at atmospheric pressure and at a temperature of 20.6° ±

0.3° C. Initial gas samples were secured in Bailey bottles as in the experiments with *Paramecium*. The tubes were then rotated on their long axes thirty to sixty times a minute at the equilibration temperature. Under these conditions the eggs were evenly distributed throughout the suspension. Cleavage proceeded in the normal manner, but it was somewhat delayed in time. A quantitative study of the retardation in cleavage at different carbon dioxide tensions will be published elsewhere by Haywood and Root (1930). Eighty to one hundred per cent cleavage was obtained in all the air tonometers except those indicated in Table V. Cytolysis during the experiments was not observed when fertilized eggs were used, although unfertilized eggs were found to be extremely fragile.

TABLE V

Respiration of Fertilized Arbacia Eggs at Different Carbon Dioxide Tensions

Experiment No.	CO_2 Tensions in Tonometer B	Respiration in Tonometer A			Respiration in Tonometer B			Ratio O_2 Consumption $\frac{B}{A}$	Ratio CO_2 Production $\frac{B}{A}$
		O_2 Consumed	CO_2 Produced	*RQ*	O_2 Consumed	CO_2 Produced	*RQ*		
	mm. Hg	*cc.*	*cc.*		*cc.*	*cc.*			
1	0.38– 2.92	0.722	0.346	0.479	0.664	0.318	0.486	0.920	0.919
2	0.30– 7.85	1.069	0.803	0.751	1.005	0.848	0.843	0.940	1.056
3	0.30– 5.03	0.816	0.504	0.619	0.762	0.550	0.722	0.934	1.091
4*	1.27– 10.07	1.285	0.963	0.749	1.161	0.844	0.726	0.903	0.876
5	1.55– 6.55	0.471	0.401	0.851	0.446	0.365	0.817	0.946	0.910
6	6.72– 10.82	1.031	0.734	0.711	0.823	0.436	0.529	0.798	0.594
7	6.97– 14.24	1.319	0.924	0.700	1.022	1.086	1.062	0.774	1.175
8	9.74– 19.77	2.406	1.564	0.650	1.755	1.178	0.671	0.722	0.753
9	12.58– 14.72	0.456	0.335	0.734	0.320	0.228	0.712	0.702	0.680
10*	16.23– 21.51	1.823	1.380	0.757	0.964	0.559	0.579	0.529	0.405
11	19.96– 25.19	1.794	1.163	0.648	0.993	0.603	0.607	0.553	0.518
12	25.40– 27.03	0.506	0.345	0.681	0.220	0.172	0.786	0.434	0.498
13	27.58– 29.28	1.154	0.848	0.734	0.426	0.175	0.410	0.369	0.206
14	34.67– 37.88	1.013	0.646	0.637	0.295	0.345	1.169	0.291	0.534
15	37.80– 40.68	1.103	0.910	0.804	0.324	0.306	0.928	0.293	0.336
16	42.33– 44.57	0.925	0.683	0.738	0.206	0.232	1.126	0.222	0.339
17	43.80– 45.07	0.608	0.359	0.590	0.173	0.131	0.754	0.284	0.365
18	51.73– 52.48	0.629	0.575	0.914	0.144	0.081	0.562	0.229	0.140
19	58.43– 60.16	1.111	0.761	0.685	0.205	0.181	0.882	0.184	0.236
20	63.93– 65.14	0.999	0.829	0.829	0.339	0.131	0.386	0.339	0.157
21	64.59– 65.49	0.883	0.629	0.712	0.187	0.101	0.540	0.211	0.160
22	71.51– 72.56	0.860	0.588	0.683	0.197	0.112	0.577	0.229	0.190
23*	85.50– 87.94	0.992	0.729	0.734	0.343	0.192	0.560	0.345	0.263
24*	86.90– 88.02	0.615	0.439	0.713	0.178	0.119	0.669	0.289	0.271
25	124.99–125.75	0.558	0.356	0.638	0.185	0.075	0.405	0.331	0.210
26	131.61–133.33	0.793	0.570	0.718	0.143	0.184	1.286	0.180	0.322
27	139.79–140.41	0.543	0.387	0.712	0.147	0.065	0.442	0.270	0.168
28*	162.92–163.15	0.972	0.775	0.797	0.176	0.024	0.136	0.181	0.030
29	176.02–177.19	0.558	0.413	0.740	0.045	0.125	0.277	0.081	0.302

* Less than 80 per cent cleavage in air tubes.

In the experiments whose duration deviated from three hours, the respiratory exchange was calculated for this time. At 20° C., the first cell division occurred about one hour after fertilization in the control tonometers. At the end of two hours the cells were in the four and eight cell stages; at the end of three hours, they were in the sixteen and thirty-two cell stages. The material was not, therefore, unicellular throughout the whole experiment. No differences were observed in the effects of carbon dioxide upon the rate of oxygen consumption in experiments in which the egg had started to cleave before introduction into the tonometers, as compared with eggs which had not started to cleave at that time.

At the end of the experiment, samples of gas were secured from both tonometers and analyzed. The amounts of oxygen and of carbon dioxide in the gas and in the sea water were then calculated for the beginning and for the end of the experiments. For this calculation, the absorption coefficients for oxygen and carbon dioxide in sea water given by Krümmel (1907) were used.

The results obtained in twenty-nine experiments are given in Table V and shown graphically in Fig. 3. It will be observed in Fig. 3

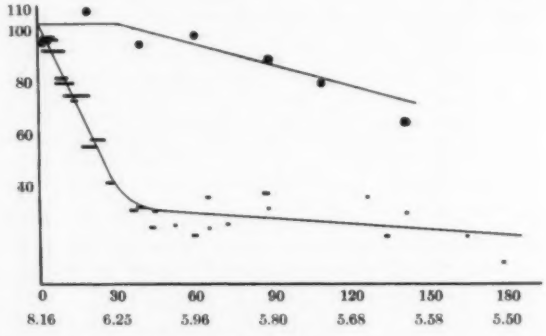

FIG. 3. The effect of carbon dioxide upon the oxygen consumption of fertilized *Arbacia* eggs.
Abscissa and ordinate as in Fig. 1.
— = carbon dioxide experiments.
● = hydrochloric acid experiments.

that, for each 10 mm. increase in the carbon dioxide tension up to 30 mm. Hg, there is a 21 per cent decrease in the rate of oxygen consumption. Above 30 mm. Hg, further increases in the carbon dioxide tension have a relatively slight effect upon the rate of oxygen consumption.

The average of the respiratory quotients obtained in air in thirty-two determinations was 0.71, which is lower than the average value of 0.78 obtained by Amberson (1928) in twenty experiments. The respiratory quotients in the presence of carbon dioxide must be interpreted with caution, for they range irregularly between 0.14 and 1.29. When the twenty-five carbon dioxide experiments in Table V are arranged in five groups of five experiments each, and the respiratory quotients and ratios of oxygen consumption and carbon dioxide production averaged, Table VI is obtained. It may be observed that, in the presence of carbon dioxide, the respiratory quotients are slightly higher than the control values up to 60 mm. Hg carbon dioxide tension. Above this tension, the respiratory quotients are lower. The carbon dioxide production and the oxygen consumption both decrease as the carbon dioxide tension increases.

TABLE VI

Summary of Experiments in Table V (Fertilized Arbacia Eggs)

Group	Experiments	CO_2 Tension mm. Hg	*RQ* Control	*RQ* CO_2 Tonometer	Ratio O_2 Consumption $\frac{CO_2 \text{ tonometer}}{\text{control}}$	Ratio CO_2 Production $\frac{CO_2 \text{ tonometer}}{\text{control}}$
I.....	5–9	1.6– 19.8	0.729	0.758	0.788	0.822
II....	10–14	16.2– 37.9	0.691	0.710	0.435	0.432
III...	15–19	37.8– 60.2	0.746	0.850	0.242	0.283
IV...	20–24	63.9– 88.0	0.734	0.546	0.282	0.208
V....	25–29	125.0–177.2	0.721	0.509	0.208	0.206

In order to determine whether carbon dioxide decreased the rate of oxygen consumption of fertilized *Arbacia* eggs by some internal effect, or merely by changing the pH of the sea water, a group of experiments was carried out in which approximately the same pH range was produced with hydrochloric acid which enters cells with difficulty, if at all. In these experiments, sea water was first made bicarbonate-free by adding concentrated hydrochloric acid to bring it to a pH of 3 to 4, and was ærated overnight. It was then returned to the desired pH by the addition of concentrated sodium hydroxide. The hydrogen ion concentration was determined by means of a quinhydrone electrode with an accuracy of 0.02 to 0.03 pH. The pH so obtained was compared with that of sea water at different carbon dioxide tensions as determined by Henderson and Cohn (1916).

The results of these experiments show a noticeable, but much smaller depression of the rate of oxygen consumption beginning at about pH 6.2 (corresponding to 35 mm. Hg carbon dioxide tension), and falling to about 62 per cent of the control at pH 5.6 (corresponding to that of sea water in equilibrium with carbon dioxide at 140 mm. Hg). (Table VII and Fig. 3.)

TABLE VII

The Effect of Hydrochloric Acid on the Respiration of Fertilized Arbacia Eggs

Experiment No.	Tonometer B pH	Resp. in Tonometer A			Resp. in Tonometer B			Ratio O_2 Consumption $\frac{B}{A}$
		O_2 Consumed	CO_2 Produced	*RQ*	O_2 Consumed	CO_2 Produced	*RQ*	
		cc.	*cc.*		*cc.*	*cc.*		
1	6.43	0.670	0.302	0.450	0.702	0.289	0.410	1.048
2	6.15	0.598	0.282	0.471	0.550	0.302	0.549	0.919
3	5.96	0.661	—	—	0.630	0.200	0.317	0.953
4	5.81	0.473	0.255	0.539	0.405	0.237	0.585	0.856
5	5.71	0.541	0.054	0.099	0.417	0.220	0.527	0.770
6	5.60	0.448	0.207	0.462	0.278	0.172	0.618	0.620

DISCUSSION

While the increased rate of oxygen consumption of *Paramecium* between carbon dioxide tensions of 15 and 66 mm. Hg probably lies within the experimental error, no experiment within this range resulted in a decreased rate of oxygen consumption. Since it is generally known that carbon dioxide causes an initial increase in the motility of *Paramecium* and other ciliates, followed by a decrease, it is suggested that the increased rate of oxygen consumption observed is a result of the increased motility of the organisms induced by carbon dioxide. This view is strengthened by the experiments performed upon *Arbacia* eggs in which there was no independent motility, and in which no increase in the rate of oxygen consumption was observed at any carbon dioxide tension.

Hydrochloric acid exerts a far less profound effect upon the rate of oxygen consumption of *Paramecium* and the fertilized eggs of *Arbacia* than does carbon dioxide at the same pH. Jacobs (1920*a*, 1920*b*, 1924) has shown that carbon dioxide far surpasses the acids which he studied in the rapidity with which its effects are produced. Hydrochloric acid, on the other hand, penetrates cells very slowly, if at all (Loeb, 1909; Jacobs, 1924). The differences in the results obtained by the action of carbon dioxide and of hydrochloric acid upon

the rate of oxygen consumption of fertilized *Arbacia* eggs and *Paramecium* may perhaps be interpreted as due to differences in the ability of the two acids to penetrate the cell membrane, and to produce within the cell changes in pH, though it is, of course, possible that the relatively profound effect of carbon dioxide upon the rate of oxygen consumption of these cells may be due to some other specific effect of its molecule.

The respiratory quotients of *Paramecium* rise progressively as the carbon dioxide tension is increased (Table III). It is possible that the suppression of oxidations under these conditions results in the production of acid metabolites which drive out carbon dioxide from bicarbonate contained in the cells and in the surrounding medium. The respiratory quotients of the sea urchin eggs, however, do not increase in the presence of high carbon dioxide tensions (Table VI). In these cells it is possible that acid substances are not produced when the rate of oxygen consumption is decreased by carbon dioxide, or that such acid substances may be rapidly reconverted into a non-acid form so that they do not accumulate in appreciable amounts. It would appear, assuming the absence of gross errors, that the respiratory reactions of the two cells to carbon dioxide differ.

I wish to express my appreciation for the interest and stimulating suggestions made by Dr. W. R. Amberson, under whose direction these experiments were performed.

Summary

1. The respiratory exchanges of *Paramecium caudatum* and the fertilized eggs of *Arbacia punctulata* have been studied by the method of gas analysis.

2. When *Paramecium* is exposed to different gas mixtures containing carbon dioxide at progressively increasing tensions, the rate of oxygen consumption increases slightly at the lower tensions and decreases at the higher tensions. The rate of oxygen consumption of fertilized *Arbacia* eggs is depressed by carbon dioxide at all of the tensions studied.

3. Moderate changes in the pH of the surrounding medium produced by hydrochloric acid have no apparent effect upon the rate of oxygen consumption of *Paramecium*. Similar changes in pH produced by hydrochloric acid diminish the rate of oxygen consumption of fertilized *Arbacia* eggs, although less markedly than does carbon dioxide at the same hydrogen ion concentration. The greater effectiveness of carbon dioxide in producing changes in the respiratory rate is presumably related to its greater penetrating power.

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A QUANTITATIVE STUDY OF THE EFFECT OF CARBON DIOXIDE UPON THE CLEAVAGE RATE OF THE ARBACIA EGG

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In two important respects carbon dioxide claims a place of peculiar interest for the physiologist. First, as a metabolic product it is present wherever there are active cells. Secondly, as many workers have observed, it has the effect of very promptly depressing certain cell activities. The exact extent of this depressant action of carbon dioxide upon the cells which produce it is an important question yet to be answered. A step in this direction, however, can be made by a determination, as quantitative as possible, of the effect of relatively low, accurately measured tensions of carbon dioxide upon some type of cell activity.

For such a study the fertilized egg of the sea urchin is especially useful, since it is less subject to wide variations than are many other cells, and since its rate of cleavage provides a convenient means of measuring any possible effects produced. Moreover, inasmuch as one of us (Root, 1930) has shown that the presence of carbon dioxide definitely limits the amount of oxygen consumed by the fertilized *Arbacia* egg, it is of interest to ascertain whether this effect of various known tensions of carbon dioxide upon cell oxidations is paralleled by its effect upon the cleavage rate.

Haywood (1927) has shown that, if carbon dioxide at a tension greater than 20 per cent of an atmosphere is added for even a few minutes to the sea water surrounding the newly fertilized eggs of *Arbacia*, cleavage suffers a distinct retardation. It might, therefore, be expected that lower tensions of the gas could also delay cleavage if administered for longer periods of time. In fact, this was earlier demonstrated by Smith and Clowes (1924) by a different method. These workers obtained the carbon dioxide from the bicarbonate of the sea water by the addition of hydrochloric acid, but with the result that the amount of bicarbonate remaining was not constant throughout this series of experiments. Since they themselves showed that the inhibitory effect of carbon dioxide is increased as the amount of bicarbonate is decreased, the results obtained by these workers are of a relatively complex nature.

In the present investigation, the newly fertilized eggs of *Arbacia* were subjected to sea water whose composition was not altered except by the addition of carbon dioxide. Moreover, we believe that our method has a further theoretical advantage because the data are based on the time required for one definite stage in the cleavage process to be reached (*i.e.*, the appearance of the first cleavage in 50 per cent of the eggs) rather than on the total number of cleavages occurring in all of the eggs in some arbitrarily selected time.

Method

Sea water containing the desired tension of carbon dioxide was prepared by equilibration with carbon dioxide-air mixtures, as described by Root (1930) elsewhere. This method consisted essentially of bubbling the gas from a mixing bottle into a tonometer containing sea water until the latter was found to be fully equilibrated with the gas, at which time duplicate gas samples were taken for subsequent analysis with a Bazett modification (1928) of the Haldane-Henderson gas analyzer. At the same time a tonometer for the control was equilibrated with room air. Our method was slightly modified from that of Root in two ways. First, both the carbon dioxide-air mixture and the room air used for equilibration were passed through a series of bottles containing sea water before entering the tonometers, to eliminate any possible change in osmotic pressure through evaporation. Secondly, the eggs were not added to the tonometers until *after* equilibration, because preliminary control experiments had indicated that the agitation of the eggs by the bubbling of the gas apparently had a retarding effect upon the rate of cleavage. Since only 0.9 cc. of egg suspension was introduced into 75–90 cc. of sea water in the tonometers which were used, it will be evident that any change in gaseous content due to the addition of the eggs was extremely small.

The lowering of the oxygen content of the carbon dioxide-air mixture caused by the addition of the carbon dioxide to room air could be entirely disregarded, for a previous study by Haywood (1927) has shown that the oxygen tension may be reduced far more than occurred in these experiments before the cleavage rate is affected by lack of oxygen.

The suspensions of eggs and sperm of *Arbacia punctulata* which were employed were freshly obtained and were freed of debris. A preliminary observation of their quality was made by fertilizing samples and noting the appearance of the fertilization membrane. The eggs were inseminated and then allowed to settle for five or six minutes. At the end of that time they were gently centrifuged by means of a

hand centrifuge and the supernatant liquid containing sperm was removed, after which the egg suspension was diluted to the desired concentration by the addition of a small amount of sea water. At exactly 10 minutes after insemination, 0.9 cc. of the resulting egg suspension was introduced into each of the two tonometers, one of which was already equilibrated at the desired temperature with a carbon dioxide-air mixture, the other, with room air. Besides the two tonometers, a glass-stoppered bottle of about 80 cc. capacity, completely filled with sea water, also received 0.9 cc. of egg suspension. This bottle was useful as a check upon the control tonometer and also allowed inspection of the eggs at times when it was not desirable to open the control tonometer.

A temperature of $20.6° \pm 0.5°$ C. was maintained by means of ice in all the experiments except those of eight hours' or more duration. In these longer experiments the variations in temperature were not more than $\pm 2.0°$ C.

After exposure to the gas of the tonometers for varying lengths of time, samples of the eggs were obtained for observation. After a preliminary precaution of cleaning the outlet tube of the tonometer with a stream of sea water, a few drops of the suspension were removed as a sample. This could be done without the admission of air by opening only the lower stop-cock while the tonometer was held in the hand for a moment, the warmth of the hand expanding the gas within the tonometer sufficiently to force out the sample. Further division of the eggs thus removed was immediately prevented by adding a weak solution of formaldehyde in sea water, which preserved them until a time when they could be accurately counted. It was, of course, necessary to take as few samples as possible in order to minimize the loss of gas from the tonometers. Four or five samples were usually sufficient to provide the requisite data, cleavage in 50 per cent of the eggs being completed in most cases before the third sample was taken. Since the samples could ordinarily be obtained within a few minutes of one another, the period during which the carbon dioxide tension was slightly lowered was a relatively short one. The maximum reduction in tension due to total sampling was never greater than 5 per cent.

The eggs were counted to determine the percentage showing the first cleavage. The counts were based upon samples of two hundred or more eggs, but occasionally it was necessary to use smaller samples. The percentages were then plotted against the time in minutes after insemination (Fig. 1). As already stated in an earlier paper (Haywood, 1927), the resulting curve theoretically represents the number of eggs which from minute to minute are undergoing the first cleavage,

and from it by interpolation may easily be determined the time required for cleavage to appear in any given percentage of eggs. The time required for the first cleavage in 50 per cent of the eggs was chosen as a convenient criterion of the rapidity of the cleavage process, and will be referred to subsequently as the *cleavage time*. A comparison of the cleavage time of the eggs of the two tonometers, therefore, gives a basis for studying quantitatively the effect of carbon dioxide upon the rate of cleavage.

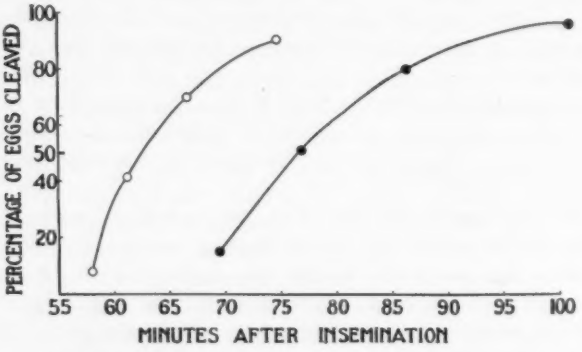

FIG. 1. The cleavage rate of *Arbacia* eggs during exposure to a carbon dioxide tension of 9.5 mm. Hg.

Abscissa = time, in minutes, after insemination.
Ordinate = percentage of eggs showing the first cleavage.
● = exposed eggs. ○ = control eggs.

Both sets of eggs were placed in the tonometers 10 minutes after insemination. Temperature = 20.6°–20.8° C.

RESULTS

The data of a typical experiment are shown graphically in Fig. 1. From the curve at the left, the cleavage time of the control was found to be 62.5 minutes, while a similar curve, unquestionably shifted toward the right, shows the effect of 9.5 mm. Hg tension of carbon dioxide in retarding the cleavage time to 76.5 minutes. This represents a delay of 14 minutes. Since exposure to carbon dioxide was not begun until 10 minutes after insemination, it must be realized that in every case the time of exposure was actually 10 minutes less than the time of cleavage. Thus, in the experiment cited, the 76.5 minutes measured as the cleavage time really represent but 66.5 minutes of exposure, and hence the value of 14 minutes given as the delay for the carbon dioxide tension employed may differ slightly from that which would

have been obtained had the exposure been started immediately after fertilization. At the higher tensions of carbon dioxide, the curves obtained were somewhat flatter, and the number of eggs ultimately cleaving was often not as great. Abnormalities of cleavage were also to be found at some of the tensions employed. In such cases, the abnormally cleaved eggs were counted separately from the normal ones, but the total number of cleavages, abnormal as well as normal, was used in determining the cleavage time.

By means of sets of cleavage curves, obtained as were those in Fig. 1, the retardation of cleavage was determined for twenty-five different tensions of carbon dioxide, covering a range of from 1.8 mm. Hg to 44.0 mm. Hg. Some variation is naturally to be expected, especially since the range of carbon dioxide tensions employed was a relatively narrow one. Nevertheless, the general trend of the data is wholly obvious, as may be seen by observing Fig. 2. The pH values were taken from the data of Henderson and Cohn (1916). Tensions of as low as 4 mm. were able to retard cleavage 9 minutes, while one of 15 mm., which corresponds to about 2 per cent carbon dioxide, delayed it 16 minutes or more. With higher tensions of carbon dioxide, up to about 35 or 40 mm., the retardation was correspondingly greater. At tensions of 35 or 40 mm. and above, small increments of the gas were found to inhibit cleavage to a degree which was proportionally much greater than at lower tensions, and the curve, therefore, ascends steeply from this point on.

Whatever may be the importance of this critical tension of carbon dioxide, the effectiveness of very small amounts of the gas in retarding cleavage seems of real significance. Since tensions of the gas corresponding to 1 or 2 per cent—values which may conceivably approach carbon dioxide concentrations under conditions of crowding—are capable of causing an easily observed delay, it is apparent that the cells are highly sensitive to this gas.

The effects of carbon dioxide upon cell division appear quantitatively quite similar to those upon cell oxidations. This may readily be seen by comparing our curve in Fig. 2 with that obtained by one of us (Root, 1930) when the oxygen consumption of the eggs is plotted against the carbon dioxide tensions of the medium. It should be noted that both curves bend sharply at relatively the same carbon dioxide tension,—namely, at 35–40 mm. Hg. Apparently carbon dioxide acts upon some system within the cell which results in a depressed rate of oxygen consumption and an increased cleavage time.

As was mentioned earlier, when eggs were found to have divided abnormally in solutions containing carbon dioxide, the total number

of cleavages, abnormal as well as normal, was used in plotting the cleavage curves. Therefore a different set of symbols was used in Fig. 2 to designate cleavage times in eggs where abnormalities resulted from exposure to the carbon dioxide. An examination of the relative number of abnormal and normal cleavages occurring at twelve different carbon dioxide tensions between 25.6 and 49.4 mm. Hg inclusive, showed the average ratio of abnormal to normal cleavages to be 2.5. The ratios ranged from 1 to 5.5 and tended to be somewhat higher for the higher carbon dioxide tensions.

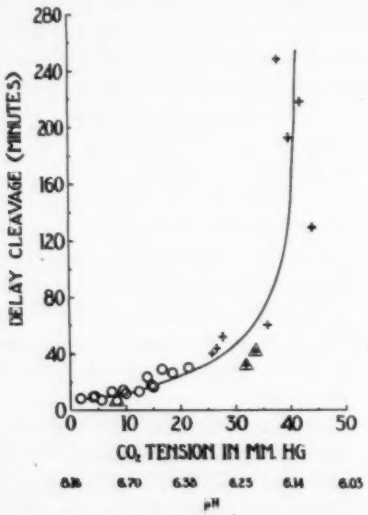

FIG. 2. The retardation of the first cleavage of *Arbacia* eggs during exposure to various carbon dioxide tensions.
Abscissa = carbon dioxide tension in mm. Hg; pH is also given.
Ordinate = minutes of retardation of the first cleavage.
○ = cleavages normal in at least 95 per cent of the cleaved eggs.
\+ = abnormal cleavages included.
△ = values approximate only, since these were obtained by extrapolation.
Temperature = 20.6° ± 0.5° C.

After determining the extent to which cleavage was retarded by relatively low tensions of carbon dioxide, we next undertook to ascertain the tension of this gas which would be required to suppress cleavage completely. In these experiments, the eggs were exposed to carbon dioxide for eight hours or more, at the end of which time samples were taken and the percentage of cleaved eggs noted. Two controls were always carefully made in these experiments: First, a count was

made of eggs which had been fixed in formaldehyde soon after fertilization, in order to make sure that no cleaved eggs were accidentally present; secondly, a count was made of eggs which had been kept for at least an hour in ordinary sea water, in order to determine whether or not the eggs used were fully capable of undergoing division. If the exposed eggs showed no cleavage in eight hours, the suppression of cleavage was regarded as complete. Such completely suppressed eggs were found to show no abnormalities in appearance, but retained completely the appearance of newly fertilized eggs. The results obtained are shown in Fig. 3, where the percentage of eggs cleaved is plotted against the tension of carbon dioxide used. Although variations in the results obtained with different groups of eggs make it impossible to designate any one tension of carbon dioxide as the threshold for the complete suppression of cleavage, the data are sufficiently consistent to allow a tentative estimate of 120 to 130 mm. Hg carbon dioxide for this value. This would correspond to 16 to 17 per cent carbon dioxide at 760 mm. Hg and 20.6° C., or a solution one-sixth saturated with carbon dioxide.

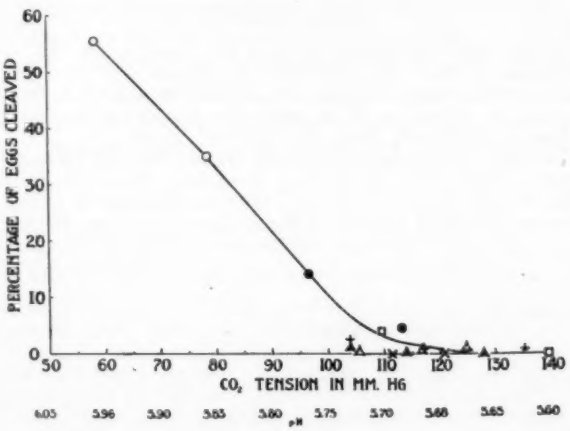

FIG. 3. The point of complete suppression of *Arbacia* eggs with carbon dioxide.

Abscissa = carbon dioxide tension in mm. Hg; pH is also given.

Ordinate = percentage of eggs showing the first cleavage after exposure of eight hours or more to carbon dioxide. Abnormals as well as normals are included.

The various symbols represent the different egg suspensions used.

Temperature = 20.6° ± 2.0° C.

This value of 16 to 17 per cent carbon dioxide, which is required for total inhibition of cleavage, is in wide disagreement with the 3.8 per cent obtained by Smith and Clowes (1924) at 20.0° C. It is important to realize, however, that the method of these investigators of liberating carbon dioxide from the bicarbonate of sea water by adding strong acid introduces a second variable—a varying bicarbonate content. These authors point out that, with a diminished bicarbonate content, the effect of carbon dioxide is more pronounced. The experimental conditions of Smith and Clowes' work differ sufficiently from those of ours to make difficult an agreement between the two sets of values.

Summary

1. Sea water equilibrated with analyzed carbon dioxide-air mixtures was found to retard the first cleavage of *Arbacia* eggs which were introduced into the mixture ten minutes after insemination.

2. A measurable delay of cleavage occurred at tensions of carbon dioxide of as low as 4 mm. Hg. Retardation was progressively greater up to 120–130 mm. Hg, at which point complete suppression occurred.

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NOTES ON THE DEVELOPMENT OF FRAGMENTS OF THE FERTILIZED CHÆTOPTERUS EGG

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In a preceding paper (1929) it was shown that fertilized fragments of the unfertilized *Chætopterus* egg may segment exactly like whole eggs, at least as far as the 16-cell stage, and may develop into swimming dwarf larvæ that are sometimes closely similar to the normal whole trochophores. The experiments here reported were undertaken in the hope of obtaining light on progressive localization in this egg prior to cleavage by comparing the behavior of fragments of *fertilized* eggs taken at successive intervals during the maturation-fertilization period, somewhat as was long ago undertaken in the case of the nemertine egg by Yatsu (1904, 1910) and by Zeleny (1904). In this respect the results of numerous experiments have been disappointing; but some of the incidental ones may be suggestive of further work.[1] No report is here offered concerning the later stages of development; but it may be stated that a certain number of swimming dwarf larvæ are produced from the fragments, among them a few that approach the normal form. Thus far, however, none have been seen that were completely normal.

I

The experiments clearly show that up to the completion of maturation, the first cleavage of fragments of fertilized eggs does not differ

[1] The method of fragmentation was for the most part the same as in my preceding work (1929), namely, by centrifuging the eggs at high speed. In that work, by an error, the speed of rotation was stated to have been about 7,000 revolutions per minute; but this was too high. In the present case the speed, though not precisely determined, was not far from half that, and the time was reduced to 3–5 minutes. The proper speed and time for good fragmentation is easily determined by a few trials. A good many eggs were also cut in two individually by the glass hair; but the centrifuge method yields fragments in much greater numbers and of greater viability. Only eggs and sperm were used that showed in controls at least 98–100 per cent of normal 2-cell stages. The statistical results were obtained mainly from material fixed with dilute osmic acid, which when properly used gives perfect preservation of external form.

In these eggs, as is well known, maturation is initiated almost immediately after the eggs are set free into the sea-water, but proceeds no further until after fertilization. The times given vary more or less with temperature and other conditions.

in any very striking way from that of fertilized fragments of unfertilized eggs. In both alike a high percentage of the fragments divide unequally in nearly or quite the same proportions as in the development of whole eggs, producing 2-cell stages that appear as miniatures of the whole eggs at the same stage.[2] At the time of maturation a slight falling off of normal 2's is noticeable, owing to a corresponding rise in the percentage of abnormal cleavages; but it is uncertain whether this is significant. At any rate, the first marked change appears suddenly in fragments from eggs centrifuged at a critical period that begins about 10–15 minutes after extrusion of the second polocyte. At this

TABLE I *

Fragments of Unfertilized and Fertilized Eggs

	Time of placing on centrifuge for 3–5 minutes	Total number of fragments	Normal 2-cell stages
1	Before fertilization	1350	1141 = 85 per cent.
2	Before fertilization	155	135 = 88 per cent.
3	12–13 minutes after fertilization	433	352 = 81 per cent.
4	25 minutes after fertilization, just before first polocyte	148	111 = 75 per cent.
5	2 minutes after first polocyte	306	207 = 68 per cent.
6	2 minutes after second polocyte	163	112 = 69 per cent.
7	10 minutes after second polocyte	155	6 = 4 per cent.

* Percentages in both tables are the nearest whole numbers. Nos. 2 and 7 are from different portions of the same lot of eggs.

time, as pointed out by Titlebaum (1928), the egg assumes a slightly marked pear-shape with the narrower end exactly at the upper pole.[3] Fragments produced by centrifuging the egg at this time show a sudden and marked drop in the percentage of normal first cleavages—often down to 10 per cent or less—and a corresponding rise in that of equal or sub-equal ones (Figs. 1–3), though a certain number of other ab-

[2] Cf. Lillie (1909), Wilson (1929).

[3] This stage was long ago figured by Mead (1898) who showed, as I have also found, that the pear-shape appears during the approach and conjugation of the pro-nuclei and persists until shortly after the fusion-nucleus breaks down. Soon after the latter event the egg again becomes spheroidal, and then again pear-shaped, but now with the narrower end towards the lower pole. Immediately afterwards the polar lobe bulges forth at the lower pole and cleavage quickly follows, about 25–30 minutes after extrusion of the second polocyte.

normal forms also are commonly seen. These facts are in part shown in Table I. In the first line, for comparison, are shown the combined results of nine control experiments, with fertilized fragments of the unfertilized egg. Lines 3–7 give the results of a series of six experiments with fertilized eggs fragmented at successive intervals during

TABLE II †

(*a*) *Fertilized Fragments of Unfertilized Eggs*

	On centrifuge	Total number of fragments	Normal 2's	Equal or sub-equal	Too unequal	"3's"	4's
1	Before fertilization	234	214 = 90%	12 = 5%	8 = 3%	0	0

(*b*) *Fragments from a single batch of fertilized eggs placed for 5 minutes on the centrifuge at the same time (rather late pear, 19 minutes after first polocyte) and then fixed in osmic acid at successive intervals for examination. The first cleavage of the fragments occurred about eight and a half minutes after release from centrifuge.*

	Time of fixation	Total number	Normal 2's	Equal or sub-equal 2's	Too unequal	"3's"	4's
2	13½ minutes after removal from centrifuge	49	18 = 37%	27 = 55%	4 = 8%	0	0
3	18½ minutes after removal from centrifuge	100	33 = 33%	56 = 56%	10 = 10%	1	0
4	27 minutes after removal from centrifuge	98	18 = 18%	12 = 12%	3 = 3%	51 = 52%	14 = 14%
5	32 minutes after removal from centrifuge	98	10 = 10%	8 = 8%	4 = 4%	62 = 63%	14 = 14%

† It is probable that many of the forms listed as "3's" in the table are actually crossed 4's seen in foreshortened view, as explained in the text. It is possible, too, that most of the 2's remaining in line 5 may have stopped developing, as often happens in these cultures.

the maturation-fertilization period. These results are typical of a much larger series except that the last item (No. 7) is selected as showing an especially low number (4 per cent) of normal 2's. As a control for this case, No. 2 shows a portion of the same batch of eggs, fragmented before fertilization, showing 88 per cent of normal 2's.

The percentages vary considerably from one experiment to another and hence are significant only within rather wide limits. Not the least

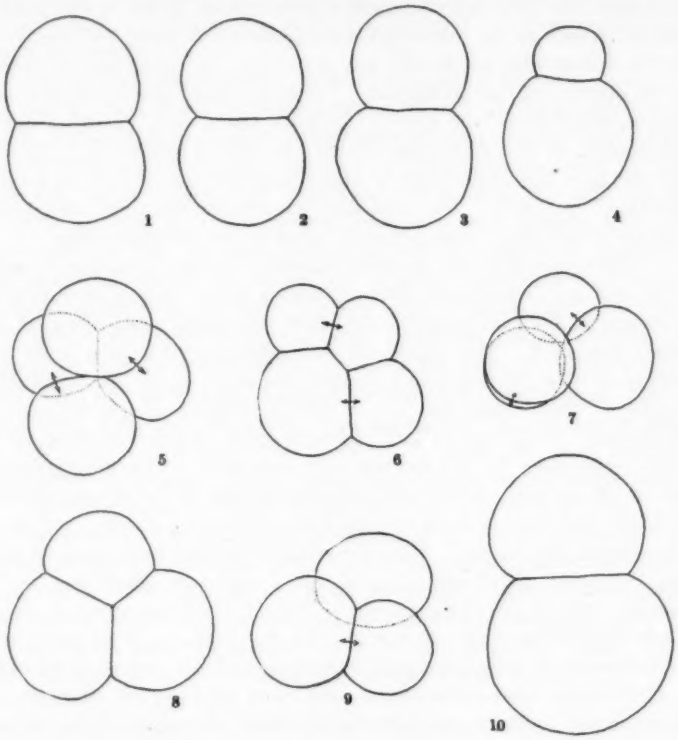
doubt, however, can exist in regard to the sudden change that takes place at the critical period (No. 7); and it is equally certain that it is in large degree due to the replacement of unequal cleavages by equal or sub-equal ones (Figs. 1–3). These results fit exactly with Titlebaum's interesting discovery (1928) that whole eggs of *Chætopterus* and *Nereis,* when compressed transversely to the axis during the pear-shaped stage, often divide equally, and that the resulting 2-cell stages sometimes produce double embryos like those observed by Penners (1926) in Tubifex.[4]

The facts are still more striking when account is taken of other forms of abnormal cleavage. A few of the fragments usually divide too unequally (Fig. 4), but with good eggs their number is always small (see Table II). Of greater interest are peculiar tripartite and quadripartite forms which closely resemble dispermic eggs (Figs. 5–9), but were finally found to arise mainly from the equal or sub-equal 2-cell stages, the second cleavage having already taken place because of delay in fixing the fragments for examination after their release from the centrifuge. It was also found that some of the tripartites arise simply by delayed division of one of the first two blastomeres, and that many others are actually 4-cell stages seen in foreshortened view, as explained below. Among the 4-cell stages are usually found a few that are normally formed (Fig. 6), as well as some that are nearly equal. In a high percentage of cases, however, and sometimes almost without exception, the 4-cell stages differ totally in aspect from the normal ones, consisting of two similar pairs of cells, each often more or less unequal, interlocking at right angles to each other to produce "crossed forms" which closely resemble those so often seen in dispermic eggs (compare Figs. 5, 6, 7). When viewed with low powers exactly in the axis of one pair, they may therefore readily be mistaken for 3-cell stages (Fig. 7).

These peculiar 4-cell forms undoubtedly arise from the 2's that precede them, and in large measure from the equal or sub-equal ones. This has been proved in a number of individual cases by following the second cleavage in life. It is shown on a much larger scale by fragmenting a large number of tested eggs from the same batch at the pear-shaped stage and dividing the products into several lots which are then fixed at successive intervals for later examination. It is obvious from the results, as partially shown in Table II, that the sudden drop in 2's and the simultaneous rise in "3's" and 4's shown at lines

[4] In an unsuccessful attempt to duplicate Penners' result in the polychæte egg I repeatedly observed in 1926 equal first cleavage in the eggs of both *Chætopterus* and *Nereis* when segmenting under pressure; but no double embryos were obtained, nor did I recognize the critical stage as such.

4 and 5 is caused by occurrence at this time of the second cleavage in most of the fragments. The considerable drop in normal 2's at the same time indicates that some of them may have produced abnormal 4's (since normal 4's are rare or absent), but this point was not sufficiently examined. In any case, it is clear that a rather high percentage of "3's" and abnormal 4's must have come from the equal or sub-equal 2's. It would be interesting to make a more adequate statistical study of this point.



FIGS. 1–10. Segmenting fragments of fertilized eggs fragmented at the pear-stage; 1–3, equal or sub-equal forms; 4, too unequal; 5, 7, crossed-type of 4-cell stage; 6, normal type; 8–9, 3-cell stages; 10, normal 2-cell stage from a whole egg. (All figures at the same enlargement.)

II

In the course of the foregoing experiments, my attention was directed to the fact that at certain stages the eggs of *Chætopterus* show

a decided tendency to orient themselves while on the centrifuge. Lillie (1906) had previously found that when unfertilized eggs containing the first maturation spindle are centrifuged at a moderate speed (1,500–2,000 revolutions per minute), so as to elongate the egg without fragmentation, the polar spindle might afterwards be found in any position with reference to the secondary stratification but most frequently lies in the hemisphere containing the gray cap (oil-cap) that is formed at the central pole of elongation. Lillie attributed this to the fact that during maturation "the force of polarity has already caused a partial aggregation of the substances before the centrifugal force is applied, and the eggs, therefore, tend to rotate with the heavier pole in a distal direction" (p. 182). He concluded nevertheless that "in a large percentage of the eggs, various causes combine to overcome this."

I have examined this question especially in eggs considerably elongated, but not fragmented, by centrifuging for a short time just after extrusion of the first polocyte, the latter subsequently affording an excellent landmark. The results show conclusively that in a large percentage of cases the polocyte is found lying either in or just outside the margin of the oil-cap that lies at the centripetal or central pole of elongation. Thus: out of 225 observed cases (4 experiments) placed on the centrifuge ½ to 1 minute after extrusion of the first polocyte, 155, or 70 per cent, showed the polocyte actually in the oil-cap, and 20, or 9 per cent, just outside its margin—*i.e.*, nearly 80 per cent in or very close to the oil-cap. This is especially noteworthy because in eggs thus elongated the oil-cap occupies but a relatively small area at the narrower end of the elongated egg. This, I think, leaves no doubt of a very marked tendency on the part of the eggs to orient themselves at this time with the animal pole turned towards the center of rotation.

Only a few other experiments were made with eggs at other stages. In one series, centrifuged immediately after extrusion of the second polocyte, 22 eggs showed 6 with the polocyte inside the oil-cap and 9 just outside it, *i.e.* nearly 70 per cent in or very near it. Experiments with eggs prior to maturation, though unsatisfactory for various reasons, point to the conclusion that the orientation is less marked than in later stages; but there are many sources of error in these cases. On the other hand, the results of centrifuging at the time of the first polocyte are so definite that this method should be useful for further experiments; for after elongation by short centrifuging the eggs may much more readily be cut in two individually and with less damage, and the plane of section may more readily be determined. Even in mass experiments at the time in question (and probably later), a rather high

probability exists that fragments containing the oil-cap are from the upper hemisphere. By these methods it should readily be possible to settle the question, among others, whether both the upper and the lower hemispheres are capable of producing complete normal larvæ (as in *Cerebratulus*), or whether (as in *Dentalium*) only the lower hemisphere has such capacity.

Light on this question may perhaps be obtained in another way. It is a striking fact that after fragmentation of the fertilized eggs during the pear-shaped stage most of the fragments that segment, and sometimes very nearly all, are the hyaline or lightly granular ones. These fragments rarely if ever divide with a polar lobe, while the small number of dark granular ones that divide commonly form such a structure. This indicates that the clear fragments are for the most part from the upper hemisphere (as might be expected also from the position of the amphiaster in the normal whole eggs), though they rarely bear polocytes. All the facts are explained under the supposition that the eggs orient themselves to a considerable extent while on the centrifuge, and hence are commonly fragmented across the egg-axis in such a manner as to separate the lower, granular region (in which presumably lies the lower pole-plasm or its homologue) from the upper one containing the amphiaster and chromosomes. The frequent absence of the polocytes from the latter fragments may very well be due to the circumstance that in continued centrifuging at fairly high speeds the region of the oil-cap at the light pole is almost invariably the first to separate from the egg, in the form of a small spheroidal fragment. By this process—which I have repeatedly observed—the polocytes would be carried off with the oil-cap in a large percentage of cases. There are here interesting possibilities for further experiment.

III

The foregoing observations do not, I fear, throw much light on the problems of localization in the *Chætopterus* egg. At first sight it may seem that the sudden change in the form of cleavage that appears in both whole eggs and their fragments after centrifuging at the pear-shaped period points to the occurrence of a corresponding crisis in the localizing process at that time—such a possibility is certainly suggested by the facts in the leeches (Whitman, Schleip) and oligochæts (Vejdovský, Penners) where the substance of the polar rings or pole-plasms is segregated, and in some measure localized, during and after the extrusion of the polocytes. It is this material—in some cases contained in the polar lobe (*Myzostoma, Ilyanassa, Dentalium*)—which during

the first cleavage passes into the posterior or CD-blastomere and thus marks out the bilaterality and antero-posterior differentiation of the embryo. Passing later, wholly or in part, into the somatoblasts (2d and 4d) it ultimately enters the teloblasts that play so important a part in building the body. In the absence of this material—*e.g.*, after removal of the polar lobe (*Ilyanassa, Dentalium*), or of the lower polar area (*Dentalium*), or in case of the isolated AB-blastomere—*Lanice, Dentalium, Tubifex*)— the embryo is unable to produce a perfect larva or, it would seem, one that is bilaterally symmetrical.[5] In the case of *Chætopterus*, however, there is as yet nothing to show whether segregation of the material in question takes place during the pear-shaped period or earlier. The latter alternative is suggested by Lillie's cytological observations on the ovarian egg (1906), by the formation of a polar lobe during the first cleavage, and by the conditions observed in *Myzostoma* and *Dentalium*. Uncertainty in regard to this point is, however, caused by the strange fact, determined by both Whitaker and myself and confirmed by my more recent observation, that after horizontal section of the unfertilized egg and subsequent fertilization, only the lower or vegetative fragment divides with a polar lobe, yet both fragments alike divide unequally and in nearly the same proportions as in case of the whole egg. Without an explanation of this puzzling fact the problem of localization in the *Chætopterus* egg will remain unsolved.

Turning aside from this question, it seems to me that the strong tendency towards equal cleavage, shown alike by whole eggs and their fragments after centrifuging at the pear-shaped stage, gives little or no indication as to whether localizing processes are in progress at the time. That tendency may most simply be regarded as a merely mechanical effect of centrifuging, similar in principle to that suggested by Titlebaum (1928) in explanation of the corresponding effect produced on whole eggs by lateral compression at the same stage. It is at this time, as is clearly seen in Mead's figures (1898) that the pronuclei are conjugating and the sperm-asters are at their maximum development (though the definitive cleavage-spindle is not fully established until a little later). Strong centrifuging at this period may simply produce a dislocation of the developing amphiaster from its normal adjustment to the organization of the cytoplasm existing at the time, more specifically, perhaps, its relation to a regional segregation of material corresponding to the pole-plasms of the leeches and oligochæts. The amphiaster, being thus liberated from its normal connections, is left free to develop as a symmetrical or homopolar structure, such as

[5] For the experimental proof of this, see Crampton (1896), Wilson (1904a, 1904c), Penners (1926).

it seems to be during the earlier period of its development, thus leading to equal cleavage.[6]

The nature of the later development might be expected to depend on the position that the amphiaster happens to take with respect to the material in question—if the latter is equally divided, symmetrical twinning might be expected; if unequally divided, various types of asymmetrical twinning might result, such, for instance, as those long ago described in earth-worms by Kleinenberg (1879) and Vejdovský (1888–1892). One is tempted to seek in the same direction some sort of clue to the peculiar 4-cell crossed type which so commonly follows equal first cleavage, and which differs in so marked and definite a way from the normal 4's (Figs. 5–7). It seems fairly clear that in the eggs of *Tubifex, Chætopterus* and *Nereis* the tendency to twinning shown after equal first cleavage involves some kind of release of the first two cells from the integrating factors which in the normal development are responsible for the production of a single embryo. Such a release, conceivably, might leave the two resulting cell-pairs free to seek the position of least mechanical resistance, *i.e.*, that of minimal contact area, which is I think that of the crossed form.

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[6] See the accurate figures of Mead (1898) which, through his courtesy, I have recently had an opportunity to compare with the original preparations. See also the corresponding accounts of Vejdovský (1888–92), of Vejdovský and Mrázek (1903), on *Rhynchelmis*, and those of Schleip (1914) on *Clepsine*.

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STUDIES OF PHOTODYNAMIC ACTION

II. The Relationship between Hemolysis by Irradiated and Non-Irradiated Eosine

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Many photodynamic substances when in sufficient concentration bring about changes in cells which are similar to the changes produced by the irradiated dye in less concentration. This effect was studied very early by Tappeiner and Jodlbauer (1904), Jodlbauer and Busck (1905) and others. Sacharoff and Sachs described hemolysis by non-irradiated erythrosine and other photodynamic substances in the paper in which they first described photodynamic hemolysis (1905). Jodlbauer and Haffner (1921*a*) summarize a certain number of such studies and point out that among photodynamic substances, those which bring about hemolysis in the lowest concentration when not irradiated are generally the most active in bringing about photodynamic phenomena in living cells. Likewise, they are most active in flocculating the colloids of hemolyzed blood cells. While this generalization has many exceptions, it indicates a correlation between the action of irradiated and non-irradiated photodynamic substances.

Jodlbauer and Haffner (1921*b*) studied the effect of hydrogen ion concentration on hemolysis by non-irradiated eosine and rose bengale (tetra-brom tetra-iodo fluorescein). They found that the hemolytic effect, as measured by the minimum dye concentration necessary to produce hemolysis, was at a minimum in the region of neutrality and increased as the reaction became either more acid or more alkaline. This increase was more pronounced on the acid side. In acid solutions of dye in high concentrations, fixation was found to take place instead of hemolysis. The fixing action, like the hemolytic action, increased with increasing acidity. These investigators did not examine the effect of hydrogen ion concentration or dye concentration on the hemolytic and fixing activity of the irradiated dyes. This has been done in the experiments described below, the results of which point to another correlation between the action of irradiated and non-irradiated dyes, namely, that the hemolytic and fixing action of the irradiated dye is modified in the same way by hydrogen ion concentration as is that of the non-irradiated dye.

Experimental

Effect of Hydrogen Ion Concentration on Hemolysis and Fixation by Irradiated Eosine.—In a preceding paper (Blum, 1930), it was shown that previously irradiated eosine may bring about hemolysis to a degree less than that occurring when the dye is irradiated together with the

Table I

Effect of Hydrogen Ion Concentration on Hemolytic Action of Irradiated and Non-Irradiated Eosine.

All solutions contain sodium phosphate buffer, isosmotic with 0.15 M NaCl. Observations made after 7 hours in dark following irradiation. H = complete hemolysis; (H) = partial hemolysis; a = cells plus eosine irradiated; b = eosine irradiated alone, cells added immediately after exposure; c = non-irradiated. a and b irradiated 1 hour (11:00 A.M.–12:00 M., Sept. 15, 1929).

Concentration of Eosine *per cent*	pH 6.0			pH 6.4			pH 6.9			pH 7.3			pH 7.6		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
1.0 (0.0014 M)	—	—	—	—	*H*	*H*	*H*	*H*	*H*	*H*	*H*	*H*	*H*	*H*	(*H*)
0.5 (0.0007 M)	—	—	—	(*H*)	*H*	(*H*)	(*H*)	*H*	(*H*)	(*H*)	*H*	—	(*H*)	(*H*)	—
0.25 (0.00035 M)	(*H*)	—	—	*H*	*H*	(*H*)	*H*	*H*	—	(*H*)	*H*	—	(*H*)	(*H*)	—
0.125 (0.00017 M)	(*H*)	*H*	—	*H*	*H*	(*H*)	*H*	*H*	—	(*H*)	(*H*)	—	(*H*)	*H*	—
0.062 (0.00009 M)	*H*	*H*	—	*H*	*H*	—	*H*	*H*	—	*H*	(*H*)	—	*H*	*H*	—
0.031 (0.00004 M)	*H*	*H*	—	*H*	*H*	—	*H*	*H*	—	*H*	(*H*)	—	*H*	*H*	—
0.015 (0.00002 M)	*H*	(*H*)	—	*H*	*H*	—	*H*	(*H*)	—	*H*	(*H*)	—	*H*	(*H*)	—
0.007 (0.00001 M)	*H*	(*H*)	—	*H*	(*H*)	—	*H*	—	—	*H*	(*H*)	—	*H*	(*H*)	—
0.004 (0.000005 M)	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—
0.002 (0.000002 M)	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—
0.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

cells. Therefore, parallel series of experiments were conducted under both conditions together with a like series, using the non-irradiated dye. The technique of the experiments was the same as that described in the above paper. A much narrower range of hydrogen ion concentration was studied than that examined by Jodlbauer and Haffner (1921*b*), as it seemed wise to avoid as much as possible the hemolytic effects of the hydrogen and hydroxyl ions themselves. Within the range here used, pH 6.0 to pH 7.7, no great difference in cell volumes occurs in

the solutions used.[1] Thus we may assume that the hemolytic action of hydrogen and hydroxyl ions is practically negligible in our experiments.

Three like series of dilutions of the dye were used at each hydrogen ion concentration; (*a*) dye irradiated together with cells, (*b*) dye irradiated alone, cells added subsequently in the dark and (*c*) dye not

TABLE II

Effect of Hydrogen Ion Concentration on Hemolytic Action of Irradiated and Non-Irradiated Eosine.

All solutions contain sodium phosphate buffer, isosmotic with 0.15 M NaCl. Observations made after 6 hours in dark following irradiation. *P* = precipitate following observation of complete hemolysis at an earlier time. Other symbols as in Table I. *a* and *b* irradiated 1 hour and 15 minutes (1:45–3:00 P.M., May 10, 1929).

Concentration of Eosine *per cent*	pH 6.0			pH 6.5			pH 7.0			pH 7.4			pH 7.7		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
4.0	—	—	—				—	*P*	*P*				*H*	*H*	*H*
2.0				—	—	—				*H*	*H*	*H*			
1.0	—	—	—				—	(*H*)	*H*				(*H*)	*H*	(*H*)
0.5				—	*H*	*H*				(*H*)	(*H*)	—			
0.25	—	—	—				(*H*)	—	—				—	(*H*)	—
0.125				(*H*)	(*H*)	—				(*H*)	—	—			
0.062	—	—	—				(*H*)	—	—				(*H*)	—	—
0.031				(*H*)	—	—				(*H*)	—	—			
0.015	—	—	—				(*H*)	—	—				(*H*)	—	—
0.007				*H*	—	—				(*H*)	—	—			

irradiated. Tables I and II are taken from typical experiments covering overlapping regions of eosine concentration, and do not agree absolutely in a quantitative sense as regards the irradiated dye. The conditions of irradiation never being the same in any two experiments, absolute agreement is never obtained, but each experiment performed demonstrates certain qualitative differences between the irradiated and non-irradiated dye. A certain parallelism may be observed in the action of the irradiated and non-irradiated dye in that, generally speaking, hemolysis occurs most readily in both cases at the same hydrogen ion concentrations; the irradiated solutions, however, show hemolysis at lower dye concentrations. The solutions irradiated alone (*b*) always appear to take a place intermediate between the non-irradiated dye (*c*) and the dye irradiated with the cells (*a*) as regards their hemolytic activity. The explanation of this latter fact has been discussed by the writer (1930).

[1] See Blum (1930).

We note in both Tables I and II, at acid reactions, that hemolysis occurs in higher concentrations in non-irradiated than in irradiated solutions. Here again series *b* appears to be intermediate between *a* and *c*. This apparently paradoxical behavior is due to fixation of the cells, as described by Jodlbauer and Haffner (1921*b*) for high concentrations of non-irradiated eosine and rose bengale at acid reactions. This is demonstrated in the following type of experiment which was carried out in the usual manner, except that after several hours in the dark following irradiation the cells were examined for fixation by treatment with distilled water. The solutions were pipetted off from all those tubes in which complete hemolysis had not occurred, leaving only the cells and debris in the bottom of the tubes. The solutions were then replaced by distilled water and allowed to stand for several hours. At the end of this time, the tubes were again examined for hemolysis, the determinations with the naked eye being checked by microscopic examination. The cells in those tubes which did not show hemolysis before or after this treatment were considered as completely fixed and are designated in Table III by the letter *F*. Those tubes which showed some hemolysis, but in which intact cells could be observed, are represented by (*F*) to indicate partial fixation of the cells. Partial fixation was also considered to have occurred in those tubes which showed hemolysis before treatment with distilled water but no hemolysis after, and these are likewise designated by (*F*). Partial hemolysis before this treatment is designated by (*H*), and complete hemolysis by *H* as in the previous tables.

This treatment of the cells is rather severe and selection of a criterion of fixation is rather arbitrary. Nevertheless, the results demonstrate in a striking manner that fixation, like hemolysis, is shifted into a lower concentration of the dye by irradiation. They also show that fixation is most apparent in both irradiated and non-irradiated dye at the same hydrogen ion concentration, and that series *b* is again intermediate between *a* and *c* as in the case of hemolysis. A consideration of the data in Table III indicates the fallacy of adopting hemolysis as a quantitative measure of photodynamic action. Very false deductions may be based upon the simple observation that hemolysis does not occur under a given set of conditions, since this may indicate either that the action of the photodynamic substance is too weak to produce hemolysis, or that it has produced fixation instead. We are obviously dealing with two manifestations of the action of eosine on red blood cells which are both modified in much the same way by irradiation and by hydrogen ion concentration.

Still a third manifestation of the action of non-irradiated and ir-

radiated dyes appears in the precipitation of cell constituents from suspensions of hemolyzed blood cells. Jodlbauer and Haffner (1921*b*) have described flocculation of hemolyzed cells by non-irradiated eosine and rose bengale. Table IV shows the results of an experiment which affords a comparison between the action of irradiated and non-irradiated eosine on hemolyzed cells. This experiment was conducted simul-

TABLE III

Fixation and Hemolysis of Red Blood Cells by Irradiated and Non-Irradiated Eosine.

All solutions contain sodium phosphate buffer, isosmotic with 0.15 M NaCl. Observations made after 7 hours and 30 minutes in dark following irradiation. *F* = complete fixation; (*F*) = partial fixation. Other symbols as in Tables I and II. *a* and *b* irradiated 2 hours and 15 minutes (October 11, 1929).

Concentration of Eosine *per cent*	pH 6.0			pH 6.5		
	a	*b*	*c*	*a*	*b*	*c*
2.0	*F*	*F*	*F*	(*F*)	(*H*) (*F*)	(*F*)
1.33	*F*	*F*	*F*	(*H*) (*F*)	(*H*)	(*H*)
0.89	*F*	*F*	*F*	(*H*) (*F*)	*H*	*H*
0.59	*F*	*F*	(*F*)	(*H*) (*F*)	*H*	(*H*)
0.39	*F*	(*H*) (*F*)	(*F*)	(*H*)	*H*	(*H*)
0.26	*F*	*H*	—	(*H*)	*H*	—
0.17	*F*	*H*	—	(*H*)	*H*	—
0.11	(*H*)	*H*	—	(*H*)	*H*	—
0.075	(*H*)	*H*	—	(*H*)	*H*	—
0.050	(*H*)	*H*	—	(*H*)	*H*	—
0.	—	—	—	—	—	—

taneously with the one described in Table II and under exactly the same conditions, except that blood cells hemolyzed with distilled water were used instead of intact blood cells. It may be readily seen that precipitation, like hemolysis and fixation, is more pronounced in irradiated than in non-irradiated solutions of the dye, and that series *b* is intermediate between *a* and *c*. Again, as with hemolysis and fixation, precipitation is most pronounced at the same hydrogen ion concentrations in both the irradiated and the non-irradiated dye.

Table V is a record from the experiment described in Table I but represents observations made five hours later or after twelve hours in the dark following irradiation. This table is inserted to show how precipitation may mask the occurrence of hemolysis, and to emphasize the importance of making more observations than one in the course of a single experiment. Tubes often show partial hemolysis at one period with subsequent precipitation of the hemolyzed cell constituents which may mask the presence of unhemolyzed fixed cells.

TABLE IV

Effect of Hydrogen Ion Concentration on Precipitation of Hemolyzed Cell Constituents by Irradiated and Non-Irradiated Eosine.

Symbols as in preceding tables. *P* = precipitate. *a* and *b* irradiated 1 hour and 15 minutes (1:45–3:00 P.M., May 10, 1929). All solutions contain sodium phosphate buffer isosmotic with 0.15 M NaCl. Observations made after 6 hours in dark following exposure of *a* and *b*.

Concentration of Eosine per cent	pH 6.0			pH 6.5			pH 7.0			pH 7.4			pH 7.7		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
4.0	*P*	*P*	*P*				*P*	*P*	*P*				—	—	—
2.0				*P*	*P*	—				slight *P*	—	—			
1.0	*P*	*P*	*P*				*P*	*P*	—				—	—	—
0.5				*P*	*P*	—				—	—	—			
0.25	*P*	*P*	slight *P*				*P*	*P*	—				—	—	—
0.125				—	—	—				—	—	—			
0.062	*P*	trace *P*	—				—	—	—				—	—	—
0.031				—	—	—				—	—	—			
0.015	*P*	—	—				—	—	—				—	—	—
0.007				*P*	—	—				—	—	—			

TABLE V

Effect of Hydrogen Ion Concentration on Hemolytic Activity of Irradiated and Non-Irradiated Eosine.

Same experiment described in Table I. Observations made after 12 hours in dark following irradiation. Symbols as in preceding tables.

Concentration of Eosine per cent	pH 6.0			pH 6.4			pH 6.9			pH 7.3			pH 7.6		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
1.0	*P*	*P*	—	*P*	*H*	*P*	*P*	*H*	*H*	*H*	*H*	*H*	*H*	*H*	(*H*)
0.5	*P*	*P*	—	*P*	*H*	(*H*)	*P*	*H*	(*H*)	*H*	*H*	(*H*)	*H*	*H*	(*H*)
0.25	*P*	*P*	—	*H*	*H*	(*H*)	*P*	*H*	—	*H*	*H*	—	*H*	*H*	—
0.125	*P*	*P*	—	*H*	*H*	(*H*)	*H*	*H*	—	*H*	*H*	—	*H*	*H*	—
0.062	*P*	*P*	—	*H*	*H*	(*H*)	*H*	*H*	—	*H*	*H*	—	*H*	*H*	—
0.031	*P*	*P*	—	*H*	*H*	—	*H*	*H*	—	*H*	(*H*)	—	*H*	*H*	—
0.015	*H*	*H*	—	*H*	*H*	—	*H*	*H*	—	*H*	(*H*)	—	*H*	(*H*)	—
0.007	*H*	(*H*)	—	*H*	(*H*)	—	*H*	—	—	*H*	(*H*)	—	*H*	(*H*)	—
0.004	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—
0.002	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—
0.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Changes in Hydrogen Ion Concentration of Eosine Solutions during Irradiation.—Since hydrogen ion concentration exerts a marked effect on the hemolytic action of both irradiated and non-irradiated eosine, changes in hydrogen ion concentration during irradiation of unbuffered solutions might be expected to markedly affect the hemolytic activity of such solutions. Such changes in hydrogen ion concentration during the course of irradiation may be readily demonstrated in dilute unbuffered solutions of eosine by means of indicators. In 0.0005 M eosine solution, the sulphonthalein indicators may be used with sufficient accuracy to observe differences of hydrogen ion concentration corresponding to 0.5 pH. The procedure in such determinations was to add the indicators to samples of irradiated and non-irradiated dye, and to compare these to buffered solutions containing the same concentration of dye and indicator. The shift of hydrogen ion concentration during irradiation was always found to be toward the acid side. In one set of experiments, unbuffered 0.0005 M eosine solutions which had a hydrogen ion concentration of approximately pH 6.5 before irradiation were found to have increased in hydrogen ion concentration to approximately pH 4.0 in the course of five hours' irradiation. The latter value is far outside the range of hydrogen ion concentrations examined in the above experiments.

Since most of the experiments of previous investigators of photodynamic processes have been carried out in unbuffered solutions, it is quite probable that such change in hydrogen ion concentration may have been a very important factor in the results obtained. It is probable that some of the failures of other investigators to produce hemolysis by previously irradiated photodynamic substances, as described by the writer (1930), may have been due to the shifting of the hydrogen ion concentration into an acid region in which fixation and not hemolysis occurs with the concentration of the dye used. The writer has, in fact, observed fixation of cells added to previously irradiated unbuffered solutions of eosine. This criticism applies to the experiments of Sacharoff and Sachs (1905), Hausmann (1909 and 1910) and Hasselbach (1909), who were unable to demonstrate hemolysis with previously irradiated solutions of numerous photodynamic substances.

Hemolysis and Fixation by Hydrogen Peroxide and Non-irradiated Eosine.—In the preceding paper of this series (Blum 1930), the probability was discussed that the alteration in hemolytic activity of eosine by irradiation is due to an increase in oxidizing power of the dye solution, resulting in the oxidation of cell constituents. From the experiments described above it appears that the effect of irradiation is simply to increase the effects produced by non-irradiated eosine, evi-

denced by the shifting of hemolysis, fixation, and precipitation into lower concentrations of the dye. This suggests that the action of the non-irradiated eosine is also an oxidation which is increased by irradiation. This is probably not the case, however, since the non-irradiated dye is not capable of oxidizing a readily oxidizable substance such as the iodide ion. Furthermore, at least certain of the changes produced by the non-irradiated dyes may be readily reversed by simply washing out the dye, (Kudo and Jodlbauer, 1908). A possible explanation of the similarity of action between irradiated and non-irradiated eosine is that irradiation brings about oxidative changes which are simply superimposed upon changes brought about by the non-irradiated dye. This assumption need not take into consideration the mechanism of the changes brought about by the non-irradiated dye.

Such an assumption can be tested to a certain extent by finding whether oxidation alone may bring about hemolysis, and whether the effect of this oxidation is increased by the presence of non-irradiated eosine. Hydrogen peroxide is capable of oxidizing iodide ion and must, therefore, have a greater oxidizing power than non-irradiated eosine. The following experiment shows the result of treating red blood cells with hydrogen peroxide and with hydrogen peroxide plus non-irradiated eosine. Series of dilutions of hydrogen peroxide and of eosine were used. The hydrogen peroxide solutions were freshly prepared from "Superoxol" (Merck) containing 30 per cent hydrogen peroxide, which was neutralized with 0.1 N sodium hydroxide and diluted with the usual mixture of primary and secondary sodium phosphates. The greatest concentration of hydrogen peroxide used in the experiments was 3 per cent, which represents a dilution of salt content of approximately 10 per cent. Much greater dilutions with water are necessary to bring about hemolysis, and as a matter of fact fixation and not hemolysis occurs at this concentration of hydrogen peroxide. In the lower dilutions of hydrogen peroxide which were used, the reduction of salt content is certainly negligible. The technique of preparation and addition of blood cells was the same as described for previous experiments. Table VI shows the result of an experiment in which the hydrogen ion concentration of the solutions was buffered at pH 6.0. It will be noted that with the peroxide solutions containing no eosine, hemolysis occurs at concentrations of 0.37 per cent to 1.5 per cent inclusive, while above this at 3 per cent fixation occurs. At the latter concentration the hemoglobin is changed to a brownish substance, probably methemoglobin. This confirms the findings of both Schmidt and Norman (1922) and Rigoni (1926). The former found that no hemolysis took place in cells treated with hydrogen peroxide (concen-

TABLE VI

Hemolysis and Fixation of Cells by Hydrogen Peroxide and Non-Irradiated Eosine

Symbols as in Table III. All solutions contain sodium phosphate buffer pH 6.0, isosmotic with 0.15 M NaCl. Observations made six hours after addition of blood cells to eosine- peroxide mixtures.

Eosine	H_2O_2										
	3.0% (0.9 M)	1.5% (0.45 M)	0.75% (0.22 M)	0.37% (0.11 M)	0.19% (0.05 M)	0.09% (0.03 M)	0.05% (0.015 M)	0.02% (0.007 M)	0.01% (0.004 M)	0.005% (0.002 M)	0.%
per cent											
1.0 (0.0014 M)	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*
0.5 (0.0007 M)	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*
0.25 (0.00035 M)	*F*	*F*	*F*	*F*	*F*	*F*	*F*	(*F*)	—	—	—
0.125 (0.00017 M)	*F*	*F*	*F*	*F*	*F*	(*H*) (*F*)	—	—	—	—	—
0.062 (0.00009 M)	*F*	*F*	*F*	(*H*) (*F*)	(*H*) (*F*)	—	—	—	—	—	—
0.031 (0.00004 M)	*F*	*F*	(*H*) (*F*)	*H*	*H*	(*H*)	(*H*)	—	—	—	—
0.015 (0.00002 M)	*F*	(*H*) (*F*)	*H*	*H*	*H*	*H*	(*H*)	—	—	—	—
0.007 (0.00001 M)	*F*	(*H*)	(*H*)	*H*	*H*	*H*	*H*	(*H*)	(*H*)	(*H*)	—
0.004 (0.000005 M)	*F*	*H*	*H*	*H*	*H*	*H*	*H*	*H*	—	(*H*)	—
0.002 (0.000002 M)	*F*	*H*	*H*	*H*	*H*	*H*	*H*	*H*	—	—	—
0.	*F*	*H*	*H*	(*H*)	—	—	—	—	—	—	—

tration not stated) but that methemoglobin formation occurred. The latter found hemolysis in only a given range of hydrogen ion concentrations, stronger as well as weaker solutions failing to bring it about. We see, thus, that an oxidizing agent alone may produce both hemolysis and fixation.

Further examination of Table VI shows that the addition of non-irradiated eosine to the peroxide greatly alters the region of the concentration at which hemolysis and fixation occur. The presence of the dye shifts the region of hemolysis and that of fixation into much lower concentrations of peroxide. Likewise the presence of peroxide shifts these phenomena into lower eosine concentration. Thus, peroxide plus eosine may accomplish hemolysis or fixation in concentrations at which neither alone is effective.

Treatment with hydrogen peroxide plus non-irradiated eosine markedly alters the solubility of hemolyzed blood cell constituents. Decrease of solubility is evidenced by precipitates varying from slight cloudiness to heavy flocculation. While the results at hand demonstrate a definite decrease in solubility at certain concentrations of hydrogen peroxide plus eosine as compared to the same concentration of eosine alone, a more careful analysis does not seem justifiable without further data.

From these experiments it seems clear that the hemolyzing and fixing effect of non-irradiated eosine may be increased by the action of an oxidizing agent such as hydrogen peroxide. We see also that oxidation alone may produce hemolysis and fixation. This evidence seems to support our assumption that the oxidations brought about by the irradiation of the dye are merely superimposed upon the action of the non-irradiated dye. Evidence was offered by the writer (1930) that in the presence of potassium iodide, irradiated eosine returns to its non-irradiated form after having oxidized a corresponding quantity of iodide ion. Similarly, we must assume that if an oxidation of cell constituents takes place, the irradiated dye must return to its non-irradiated form after having oxidized these substances. Thus we may consider the dye as playing a dual rôle, first as the non-irradiated dye, and secondly as an oxidizing agent produced by irradiation.

As shown in the above paper, the oxidizing power of previously irradiated eosine is never greater than the molecular equivalency of the dye. Consequently, we should expect that if the irradiated eosine has an oxidizing power of the same order as hydrogen peroxide, the two substances should bring about hemolysis in approximately the same concentration if the effect of the non-irradiated eosine is neglected. On the basis of the above hypothesis the hemolytic effect of the pre-

viously irradiated eosine is a summation of the oxidation and the effect of the non-irradiated dye, and hence, we should compare the irradiated eosine to peroxide plus an equal concentration of the non-irradiated dye. Comparison of Tables I and VI shows, however, that the concentrations of hydrogen peroxide required to produce hemolysis are many times greater than the concentrations of previously irradiated eosine which produce hemolysis under comparable conditions. Table I shows that at pH 6.0, previously irradiated eosine brings about hemolysis in concentrations as low as 0.00001 M. On the other hand, Table VI shows that, at the same hydrogen ion concentration, 0.002 M hydrogen peroxide is required to bring about partial hemolysis in the presence of 0.00001 M non-irradiated eosine. At slightly lower peroxide concentrations, hemolysis no longer occurs in any eosine concentration which alone does not produce hemolysis. It would thus appear that irradiated eosine is actually a much more powerful oxidizing agent than hydrogen peroxide. Noack (1920) has found, similarly, that irradiated eosine was more effective in oxidizing the sap of *Aloe soccotrina* than was hydrogen peroxide.

Discussion

The correlation made by Jodlbauer and Haffner (1921*a*) between the photodynamic activity of substances and their ability to hemolyze red blood cells in the dark, is readily explained on the basis of the hypothesis outlined above. Since the total observable effect produced by irradiated dyes would be a summation of the effect of the non-irradiated dye and the oxidative changes brought about concomitant with irradiation, an increase in the first of these factors would result in an increase of the total effect. Thus, other factors being equal, the substances having the greatest hemolytic activity in the non-irradiated state should also show the greatest hemolytic effect when irradiated.

Jodlbauer (1926) suggests a quite different explanation of this correlation. He assumes that photodynamic action is dependent upon adsorption, on the basis of the work of Jodlbauer and Haffner (1921*b*), which indicated that the hemolytic action of non-irradiated eosine and rose bengale is dependent upon an adsorption process. He suggests (1) that the dye must be adsorbed by the cells, and (2) must retain its ability to be activated by light while in combination with the cell, thus bringing about oxidative changes. Only those dyes capable of both are photodynamically active. Those dyes which are adsorbed most readily are the most active in bringing about hemolysis in the dark, but all of these do not meet the second qualification. Thus a large number of exceptions to the general rule are accounted for. However,

the hemolysis of red blood cells by previously irradiated fluorescein dyes, described by the writer (1930), renders Jodlbauer's assumption untenable, since contact of the cells and dye during irradiation is not requisite in order to produce hemolysis. The intimate contact between cells and photodynamic substance may assist in the reaction when cells and dye are irradiated together, but offers no explanation for hemolysis when the dye is separately irradiated. The hypothesis presented above offers a more satisfactory explanation of the correlation between the action of the irradiated and non-irradiated dye, in consideration of these facts.

The fact that non-irradiated photodynamic substances may produce definite changes in living cells, has been apparently disregarded by a large number of the investigators of photodynamic action. It seems, however, very important to keep this fact constantly in mind in the interpretation of photodynamic processes. This is particularly true, if we consider the effects of irradiation to be superimposed upon the effects of the non-irradiated dye as here suggested. Whatever the nature of the action of the non-irradiated eosine in producing hemolysis and fixation, we are certain that it may precipitate cell constituents, as shown by the formation of precipitates with hemolyzed cells. The fact that hemolysis and fixation are shifted in the same way by hydrogen ion concentration and by irradiation as is precipitation, suggests that the three processes are closely connected, and the possibility that the two former may be dependent upon changes in the solubility of cell constituents. At any rate, such alterations in solubility of cell constituents must be considered as a possible result of the treatment of biological substances with eosine and other photodynamic substances. We must also consider the possibility of oxidation and of changes in hydrogen ion concentration when biological substances are subjected to the action of irradiated solutions of these photodynamic agents. It is hardly possible to imagine three factors better calculated to alter cell processes than these. It seems, therefore, that until much more careful studies of the varied photodynamic phenomena are made, we need not seek farther for their explanation. It is at least unnecessary to assume unknown processes until the possibilities of the three above-mentioned clearly demonstrable factors are exhausted.

Summary

1. Hemolysis is not the only manifestation of the photodynamic effect of eosine on red blood cells, fixation and precipitation of the

hemolyzed cell constituents also occurring at proper concentrations of the dye.

2. Hemolysis, fixation, and precipitation of the cell constituents are brought about by non-irradiated eosine at proper hydrogen ion concentration and concentration of the dye. Irradiation shifts the region of occurrence of these phenomena into lower concentrations of the dye.

3. In unbuffered solutions of eosine the hydrogen ion concentration increased in the course of irradiation.

4. Hydrogen peroxide may produce hemolysis and fixation in proper concentrations.

5. Hydrogen peroxide and eosine may reënforce each other in producing hemolysis and fixation.

6. Photodynamic action may probably be regarded as a summation of the effects of the non-irradiated photodynamic substance together with the oxidative changes brought about by this substance due to irradiation.

7. Oxidative changes in cell constituents, and changes in hydrogen ion concentration, together with the change in solubility of cell constituents brought about by the photodynamic substance, may possibly account for all the phenomena generally classed under the terms *photodynamic action* or *photodynamic sensitization.*

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REACTIONS OF *CERCARIA HAMATA* TO LIGHT AND TO MECHANICAL STIMULI

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The swimming behavior of a holostome larva, *Cercaria hamata* Miller, 1923, which McCoy (1928) found to penetrate sunfishes and develop in them to the meta-cercarial stage, has been studied, as well as the effect of light upon its activity and its reactions to shadow and to mechanical stimuli. Great variety in the swimming behavior and reactions of larval trematodes, particularly to shadowing, has been found among more than fifty species studied in more or less detail by one of us, (Miller, 1927–1930). In view of the meager knowledge of the life histories of these trematodes any statement concerning the adaptive value of the behavior of any species would be mere speculation.

In the development of the work certain facts of general physiological interest were found, and the results of preliminary studies are reported in this paper. The most outstanding is the fact that a dual mechanism of stimulation is involved in the response to mechanical stimulation and to a change of light intensity (shadow).

MATERIAL AND METHODS

The snail host of *C. hamata* is *Planorbis trivolvis* Say. Snails from Ramona Lake, St. Louis County, were isolated in water in shell vials; cercariæ emerged, usually in large numbers, into the water from those snails in which the infestation was mature. The snails were easily kept alive in the laboratory and were isolated from time to time to secure cercariæ. It is assumed that these cercariæ, which are fully formed and emerge spontaneously, constitute a uniform material for experimental purposes. They live approximately 48 hours after emergence, during which period they do not feed or reproduce; hence these factors do not enter into their behavior.

Observations and experiments were made in the laboratory before a window, using diffuse daylight or sunlight, and were also carried out in a darkroom. The emerged cercariæ were placed in glass spectroscope absorption cells ($40 \times 40 \times 20$ mm.) or in small cells of various sizes made from high grade glass slides and DeKhotinsky cement. All observations were made with a binocular microscope held in a horizontal position.

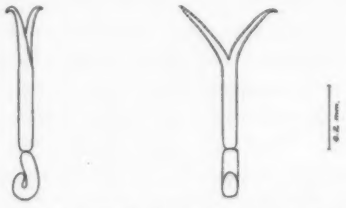
Intervals of five minutes or longer were measured with a "Reminder" clock and one minute intervals by the aid of an Eastman timer, the hand dipping into mercury and making contact every minute with a buzzer circuit. Short intervals were measured with a split-second timer registering tenths of seconds.

The light source in the darkroom was a 115 volt-200 watt, concentrated filament Mazda lamp in a 200 cm. beaver board tunnel, painted flat black. A camera shutter with a 1 inch opening was installed in the end of the tunnel. The time-setting lever was connected to an electromagnet and was operated by tapping a key. Observation in the "dark" was made by means of a weak red darkroom light behind the cell containing the cercaria. A large water cell was placed in front of the red light to absorb any heat given off.

In experiments where temperature of the water was controlled, the cell containing cercariæ was placed in a water bath and a mercury thermostat was used, connected with a heating coil and a 6-volt storage battery.

Swimming Behavior

Cercaria hamata is an intermittent swimmer. Ordinarily a short rapid upward swim alternates with a long period of sinking. The path of a swim may be changed suddenly and erratically, and occasionally an individual will make an uninterrupted swim lasting as long as 6 seconds, with many changes in direction. The tail is directed forward in locomotion, and lashes vigorously. At the end of a swim the anterior third of the body bends ventrally and posteriorly, so that the body is in the form of a hook (Text-figure), and as a cercaria sinks it



Diagrams of lateral and ventral views of *Cercaria hamata* sinking through water.

slowly comes to a position with the body down. The body retains this hook-shape until swimming is suddenly resumed. At the beginning of a sinking period the forks of the tail are spread at an angle of slightly less than 180°, but this angle gradually decreases until it is about 90°.

A great deal of variation was found among individual cercariæ in the length of a swim and of the succeeding sinking period. Ten alternating periods of sinking and swimming were measured for 75 cercariæ; sometimes a long swim followed a long period of sinking, other times a short swim followed, and thus there seems to be no correlation between them. Tables I and II show the ten observations on one individual and the averages for 10 others.

TABLE I

Variation in periods of passive sinking and of alternate spontaneous swims of one cercaria.

	Period of sinking	Duration of following swim
	seconds	*seconds*
	25.2	2.2
	48.0	3.2
	34.0	2.2
	41.0	3.0
	45.0	1.8
	107.0	2.4
	20.2	1.2
	55.6	1.0
	50.4	1.2
	39.4	3.8
Average	46.6	2.2
Range	20.2–107.0	1.0–3.8

TABLE II

Averages of periods of passive sinking and of alternate spontaneous swims of ten cercariæ.

	Average period of sinking	Average duration of swim
	seconds	*seconds*
	31.0	2.4
	45.0	1.4
	42.8	0.8
	42.7	1.2
	48.6	1.3
	80.1	1.2
	39.5	1.2
	57.1	1.2
	79.0	0.9
	26.9	1.2
Range	26.9–80.1	0.8–2.4

Effects of Temperature on Activity

The temperature of the water was noted in every case; it was found that the average period of swim did not change significantly in warming the water from 18° to 35° C., but the length of the period of sinking decreased, so that swims were more frequent at the higher temperatures.

Below 15° C. swimming behavior varied from the preceding account. The body was usually held extended when the cercaria came to rest, instead of bending. In place of swimming the cercaria exhibited jerking and lashing movements; the body sometimes bent and unbent, and occasionally, held at full length, lashed back and forth. Convulsive twitching of body and tail occurred, and the forks of the tail jerked separately. When the cercaria swam, it was only for a short distance. One individual kept up the twitching movements continuously for over 21 minutes, after which it sank to the bottom. Below 7° C. the cercariæ were either motionless on the bottom or jerked about only occasionally. Above 38° C. the body of the cercaria was held extended, but twitching did not occur.

Effect of Light on Swimming

It was observed that *C. hamata* swam more frequently in strong than in weak light; this was later tested in a darkroom. Thirty groups of ten cercariæ each were used; they were placed in a small cell and dark-adapted for an initial period of 20 or 30 minutes. During the entire experiment a weak red observation light was burning. After the cercariæ were dark-adapted, the number of swims during a one minute period was counted, and then light admitted by means of the shutter and the number of swims during a one minute period in the light was counted. These procedures were repeated, with five minute periods of dark-adaptation followed by the admission of light of different intensities. In each case the number of swims in the "dark" and in the light was counted. The following approximate light intensities, obtained by placing a 115 volt-200 watt lamp at different distances from the cell, were used: 2000, 1550, 1100, 650, 200, 100, 50 meter candles. Each group of ten cercariæ was subjected only once to each light intensity; the different intensities were used in alternation of low and high intensities so that the factor of progression would not enter.

The summary of observations on the 30 groups of cercariæ is shown in Table III. The average number of swims at each intensity was compared with the average number during the preceding one minute period of dark, and the percentage of increased activity calculated.

Reaction to Shadowing

When *C. hamata* is in a resting state, it is usually stimulated to swim, instantaneously, either by the shadow of an opaque object or by other means of decreasing the light intensity. It was thought that when failure to swim occurred it might be due to the fact that the stimulus was applied just at, or too soon after, the cessation of a spontaneous swim. Experiments were carried out on about forty individuals to test this hypothesis. A shadow was thrown upon a cercaria at the instant that it ceased swimming, and at varying intervals (1 to 10 seconds) after the cessation. Each cercaria was allowed to swim spontaneously twice between trials. A great deal of variability was found. Some individuals almost invariably swam when shadowed as soon as they came to rest; but in general the longer the interval between cessation of swimming and stimulation, the more certain the response.

Table III

Effect of Light on Frequency of Swim of Cercaria hamata

Thirty groups of 10 cercariæ used. Total number of swims during one minute of dark and during a succeeding one minute period in light of a given intensity counted, and percentage of increased activity in the light calculated. Five minute intervals between trials. High and low intensities used alternately; each group of cercariæ exposed only once to a given intensity.

Light Intensity in Meter Candles..	2000	1550	1100	650	200	100	50
Average Per cent of Increased Activity.....	626	585	408	228	94	12	8

When a shadow is thrown upon a cell containing a large number of cercariæ, concerted, or almost concerted, swimming results. *C. hamata* is stimulated to swim by touch and by currents of water, and these stimuli, occasioned by individuals which responded to the shadow, are undoubtedly factors in the general response. When the cercariæ are isolated these factors of mechanical stimulation are eliminated.

Cercariæ of this species are inhibited by repeated shadows, after responding to the first or second of the series, when an interval of one or two seconds intervenes between shadows. After a lapse of a minute or more almost all of the cercariæ swim again when shadowed, and again may be inhibited by repeated stimuli. This may be shown by the following experiment. The number of swims made by five cercariæ during 90 seconds was counted, and then a shadow cast on

the cercariæ every second and the number of swims during 90 seconds again counted, and the procedures repeated. The first shadow always brought about a response on the part of many individuals; this initial response was ignored and the number of swims in the ensuing 90 seconds counted. The data are shown in Table IV.

The effect of temperatures through a range from below 10° C. to 35° C. was studied in reference to response to shadowing; isolated cercariæ and groups of five individuals were used. Below 10° C. only an occasional cercaria swam when shadowed; from 10° C. to 35° C. the curve is practically a straight line, with 10 per cent of the cercariæ responding at 15° C. and 70 per cent at 35° C.

TABLE IV

Showing the inhibiting effects of repeated stimulation (shadowing) on the activity of C. hamata.

	Number of swims of 5 cercariae, counted in alternate 90 second periods							
Not stimulated	24		26		27		23	
Cercariae shadowed every second; swims in response to the first stimulus not counted		2		7		5		6

RESPONSE TO MECHANICAL STIMULATION

C. hamata when sinking quietly may be stimulated to swim instantly by mechanical stimuli, such as touch on any part of the body or tail, or jarring the container, or a fine stream of water. Whereas repeated stimulation by shadowing at short intervals resulted in failure to swim, it was found that this cercaria may be kept in almost continuous locomotion by repeated stimulation by touch. Forty cercariæ were used. Each was isolated in a small cell and touched twenty-five times with a fine wire on the body, tail-stem, or a fork of the tail.

In the case of twenty cercariæ the stimulation was applied as soon as possible after the individual had stopped swimming. Only seventeen failures to respond occurred in the total of 500 trials; twelve of the cercariæ swam every time. The other twenty cercariæ were permitted to assume a vertical sinking position before being touched; this allowed them an average recovery period of about 15 seconds. Thirty-five failures to swim occurred during 500 trials; seven of the cercariæ had a perfect response record for the twenty-five trials.

Several other individuals were repeatedly stimulated until fatigued.

One of these, allowed to resume the vertical sinking position between stimuli, responded 120 times and then failed to swim when stimulated ten times at short intervals. Another was touched " immediately " upon cessation of each swim. It responded to 79 successive stimuli, but after the fiftieth trial the swims became shorter and near the end of the trials the reaction consisted only of a few vibrations.

From these data and those on response to shadow stimuli, it seems clear that two different mechanisms are involved; a relatively long interval is necessary to secure anything like a regular response to the shadow stimulus, when repeated, whereas a very brief interval is sufficient in the case of stimulation by touch.

Effect of Temperature.—Between 20° C. and 38° C., the cercariæ usually swam when touched by a needle, but at lower temperatures a high percentage of failure occurred. Below 10° C. the response consisted of the jerkings which are the only movements found at these temperatures.

TABLE V

Comparison of duration of spontaneous swim with that following stimulation by shadow, touch, and stream of water. Duration of swimming in seconds.

	Cercaria *a*	Cercaria *b*
Unstimulated	1.16 ± 0.17	0.69 ± 0.07
Stimulation by:		
Shadow	2.15 ± 0.16	1.84 ± 0.17
Touch	3.24 ± 0.29	1.95 ± 0.16
Stream of Water	2.66 ± 0.24	2.01 ± 0.16

COMPARISON OF SPONTANEOUS WITH STIMULATED SWIMMING

It was observed that when *C. hamata* swam spontaneously, the average duration of swim was shorter than when it was stimulated by any of the methods used. This was tested experimentally on a number of individual cercariæ, in the following manner. The duration of a spontaneous swim was measured, and then fifteen seconds after the cercaria had stopped a shadow was cast upon it and the duration of the resulting swim measured. This was followed by touch with a fine wire and stimulation by a fine stream of water from a pipette, and the series was repeated twenty-five times for each individual. The cercaria was always allowed to swim twice between stimuli and the stimulus was then applied 15 seconds after the cessation of the second swim; occasionally the duration of the period of sinking was shorter than 15 seconds, in which event three spontaneous swims intervened between stimuli.

Data for one hundred observations on each of two cercariæ are given in Table V. It was calculated that the difference between the

mean for the spontaneous swims and those for the three types of stimuli is 4.2 (shadow), 6.2 (touch), and 5.1 (stream of water) times the probable errors in the case of cercaria *a*, and 6.2 (shadow), 7.2 (touch), and 7.4 (stream of water) in the case of cercaria *b*. For nine other cercariæ the probable errors were not calculated, but it is evident that the differences are statistically significant.

Discussion

Reactions of the sort described in this paper have been found in some species of most of the large groups of animals, but among the larval trematodes they are quite common, particularly the response to shadowing. The extent to which these varied responses aid cercariæ to reach an intermediate or final host has been studied in only one instance. Miller and McCoy (1929; and in press) have shown that the reaction of *Cercaria floridensis* to shadows, cast by fish intermediate hosts, carried the cercaria to the upper levels of the water and hence possibly was a factor in the infestation of the fishes. On the other hand, Cort and Brooks (1928), who studied the characteristic behavior but not the reactions to stimuli, of a number of fish-penetrating holostome larvæ, believe that the activity of the fish intermediate hosts is such that they would come in contact with the cercariæ, so that there is no necessity for activity on the part of the latter. Until experiments have been carried out on each species, the adaptive value of these reactions to shadow and to mechanical stimuli cannot be determined. The reactions to shadow described for *C. hamata* could be brought about in nature by animals swimming above the cercariæ, and the swimming upward would carry the cercaria toward a possible host. Judging from Cort's studies (1922) on the escape of cercariæ from their snail hosts it is likely that *Cercaria hamata* would emerge in but small numbers at temperatures below 17° C., and, on the basis of the present data on effect of low temperatures, would only occasionally react to a shadow cast by a possible host.

From the results of the present preliminary study it seems evident that a dual mechanism is involved in the response to shadow and to touch, because the cercaria may be kept in almost continuous locomotion by touch stimuli, whereas a relatively long interval must intervene between shadow stimuli to secure anything like a regular response. Experiments are now under way which are so planned as to elucidate further the nature of this dual mechanism. Effects of curare, nicotine, and other substances which specifically affect myoneural junctions are being studied, as well as the effects of polarizing currents, ultra-sonic radiation, and other forms of stimulation.

SUMMARY

1. *Cercaria hamata* swims intermittently. A short, rapid swim alternates with a relatively long period of quiet sinking.

2. The duration of the period of sinking decreases with rise in temperature. Below 15° C. the cercariæ exhibit jerking and lashing movements in place of swimming.

3. Shadowing a dense group of cercariæ results instantly in the almost simultaneous swimming of most of them. The response is partly due to the shadow stimulus and partly to the mechanical stimulus occasioned by the colliding of active individuals with quiet ones.

4. Repeated shadowing at short intervals results in inhibition of swimming.

5. Mechanical stimulation by touch (or a stream of water) initiates swimming instantaneously. Only a very short interval between stimuli is necessary to keep a cercaria in almost continuous locomotion. This indicates that a dual mechanism is involved in response to shadowing and to touch.

6. The duration of swim in response to shadow, touch, and stimulation by a stream of water is significantly greater than that of a spontaneous swim.

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ON TEMPERATURE AND THE BREATHING RHYTHM OF *CANIS MUSTELUS* AND *SQUALUS ACANTHIAS*

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The casual observation of dogfishes in aquaria reveals immediately a noteworthy regularity in the rhythmic movement of the gill-slits. The movements of the spiracles are coördinated in a perfect way with those of the slits and they go on with the same regularity, without interruption, for long periods. All these features seemed to point to the suitability of this animal for a study of the frequency of gill movements as controlled by temperature.

The previous work of Bethe, Baglioni, and others had shown considerable irregularity in the Q_{10} ratio (*cf.* Kanitz, 1915); the observations of Crozier and Stier (1924–25) on the opercular breathing rhythm in the goldfish, on the contrary, showed regularity in this rhythm under properly controlled conditions and demonstrated the applicability of the Arrhenius equation to the data obtained. Would the dogfish obey the same rule and in a similar way?

For the observations, young specimens (25 to 37 cm. long) were kept in a large aquarium the temperature of which was maintained constant to within $\pm 0.05°$ C. for periods of 30 to 50 minutes at any given temperature. The number of points on the thermometric scale chosen for the observations was made as large as possible, and in general at each temperature from 20 to 30 series of 20 beats were timed with a stop watch. The regularity of the breathing rhythm at constant temperature is remarkable; countings of 20 to 30 successive series of 20 beats often agreed to within 1 to 2 per cent. The only exception seems to be around 15° C. where more erratic values are found. We will come back later to this point. Let us note also that Crozier and Stier have found agreement of the same order of magnitude. Care was taken to allow time for complete temperature equilibrium between the organism and the medium, the water of the aquarium also being stirred gently. The aquarium was almost completely insulated by a cover which cut out most of the light; a small portion of the surface only was left uncovered to permit observation. The water in the aquarium was kept as close as possible to saturation with air, so as to exclude any possible effect of O_2 concentration. Observations made

with waters having different concentrations of O_2 did not show any direct effect of oxygen concentration upon frequency of breathing movements, at least in the range considered. The first observations showed immediately the importance of having the animals perfectly quiet. Measurements can only be made if the animals have not been actively swimming for at least five minutes, the gill rhythm being speeded up after swimming. This is in harmony with the observations of Parker (1909) ". . . that the rate of gill movement in the dogfish depends upon the momentary state of movement of the animal. When resting they vary from 35 to 40 movements per minute. When swimming slowly they respire 50 to 55 times per minute. In vigorous swimming, the rate is doubtless still more rapid." These observations were made probably between 15° and 18° C., and the frequencies agree fairly well with those here found.

The first indications given by the observations show that the values for Q_{10} are rather erratic, as will be seen in Table I.

TABLE I

Q_{10} ratios

$\frac{11.4° C.}{21.5° C.} = 2.06$	$\frac{12.70° C.}{22.75° C.} = 2.38$	$\frac{13.5° C.}{23.5° C.} = 1.72$
$\frac{14.5° C.}{24.5° C.} = 1.78$	$\frac{15.5° C.}{20.5° C.} = 1.21$	$\frac{17.5° C.}{22.75° C.} = 2.10$

The data were treated to determine the goodness of fit of the Arrhenius equation (Fig. 1). The graph shows immediately that nearly all the points are distributed within three main bands, each with parallel edges; a fourth, small, short band parallel to one of the three main ones, contains the rest of the points. These bands may be called *I, Ia; II, III* (*cf.* Fig. 1). The values found for μ are:

I and *Ia*	16,400
II	8,200
III	35,000

The first of these values corresponds exactly with that found for the opercular breathing rhythm of the goldfish by Crozier and Stier (1924–25). But, differing from what was found in the latter case, where only one temperature characteristic was secured, two additional values of μ appear (8,200 and 35,000). One should note the fact that the observational points which fall in *Ia*, were followed suddenly by points falling in the upper part of band *II*, without any smooth transition. The absolute values for the points in *Ia* are very different from those in *I*, but the slopes of both *I* and *Ia* are identical.

Another shift of points occurs also for *I*, where under certain not well-determined circumstances, in the neighborhood of 14.5° C., a sudden shift to *II* may be found. These cases of shift from one band to another are analogous to the ones found by Crozier and Stier (1925–

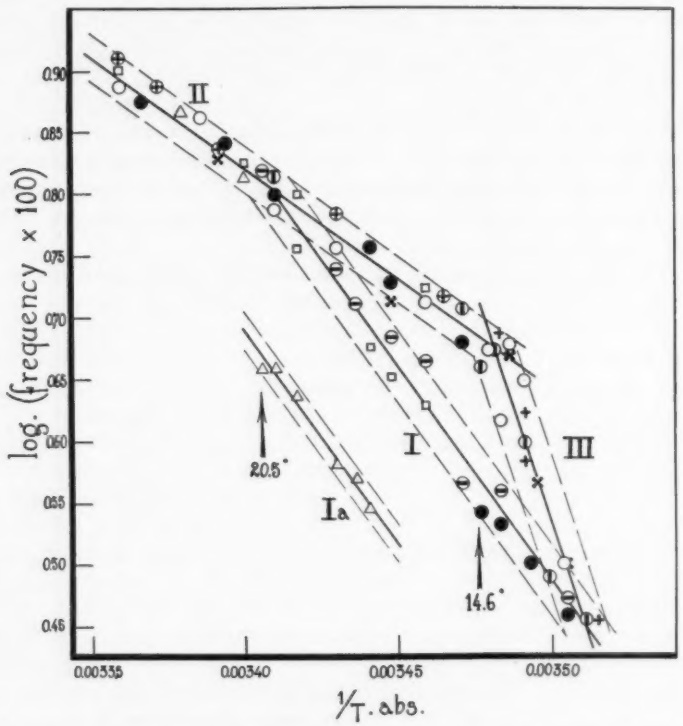

FIG. 1. Mass plot of all observations on *Mustelus canis*. Each animal has been represented by a different symbol. Each plotted point corresponds to the mean of 10 or 20 sets of readings, each reading being the time required for 20 gill movements. The number of sets of readings has been kept consistently 10 (or 20) for each animal; in other words, for any one animal there are 10 (or 20) sets of readings at each temperature.

26, p. 552) with the goldfish, after the animal was kept for 3 hours at 25° C. They agree with records obtained in many other instances, and are therefore an excellent argument in favor of the idea of different reactions playing definite rôles within definite limits of temperature. Occasionally one finds some observational point departing totally from the rest of the collection, and in many cases these data fall exactly in

the other band. These abrupt departures are especially frequent around critical temperatures, making these points very obvious in the graph when all observations are plotted separately. Necessarily all illegitimate averaging smooths out such points, which at the same time often lowers the absolute value of these average frequencies.

Changes of slopes of this nature are not surprising, as they have been found previously. They may perhaps indicate here that the " fundamental mechanism " determining the frequency of gill movement, which yields $\mu = 16{,}400$, may be in its turn linked with processes having lower (8,200) and higher (35,000) values of the critical increments. It has been suggested by Crozier and Federighi (1924–25) that the value $\mu = 8{,}200$ may be perhaps understood as reflecting the velocity of central nervous processes concerned in respiratory movements of fishes and other vertebrates. In all cases we may consider that the " fundamental mechanism " is by no means a simple one but must be probably of catenary type, more or less complicated, but where one of the three reactions (here characterized by their respective values of μ) plays the rôle of master reaction at any one moment.

We have not tried to find out any possible correlation existing between breathing and cardiac rhythms, as this would have obliged us to introduce in the animal pieces of apparatus which undoubtedly would have altered the breathing rhythm at least. We had noticed that the placing of a small glass rod (which has to be applied with utmost care in order to avoid all indirect stimulation) in the immediate vicinity of the opening of the spiracles affected singularly the coördination existing between spiracles and gill slits. Occasionally " beats " of the spiracles (in certain cases 3–4–5 in succession) are skipped although the gills operate at their normal constant rhythm. Scott (1913) had noticed also the relative independence of heart and gill movements, where he (p. 61) notes that the heart can continue to beat long after respiration has ceased, and that even when both organs are in action their rates may be increasing and decreasing independently of one another. On the other hand, Lyon (1926, p. 282) says that " even without artificial respiration, the heart may continue active long after breathing ceases " and on page 283 " that the heart rate of the sand shark is intimately related to the respiration rate. Usually the two are equal . . . the heart . . . normally takes its rate from respiration." One must remember that these experiments were performed with the sand shark (*Carcharias*), which has a slightly different type of water movement path than *Mustelus canis,* and that furthermore, as these animals were operated upon to introduce canulæ and connecting solutions for blood pressure determinations, and were kept partly out of water, they were

far from being in a state of " no interference " to which we wished to restrict ourselves.

The respective positions of the bands in Fig. 1 determine at least two critical temperatures: *I* and *II* intersect at about 20.5° C.; *II* and *III*, at about 14.5° C. The fact that these temperatures occur as critical temperatures in other respiratory activities (*cf., e.g.,* respiration of *Molge, Rana, Bufo* for 15° C., Crozier, 1924, p. 198; respiration of *Vicia faba* for 20.5° C., Navez, 1929, p. 656) is rather striking. Crozier and Stier had noticed also that at temperatures above about 15° C., the pectoral fins of the goldfish were rather active, affecting by their movements the breathing rhythm, and that below this temperature no such disturbing activity was exhibited. We pointed out previously that the latitude of variation of the data was rather small, of the order of 1 to 2 per cent. Only in the vicinity of 14.5° C. do we get a variation which is, at its maximum, 7 per cent, but which, on both sides of this temperature, reduces to its normal size. The fact that we did not get such changes in the latitude of variation for the other critical temperature seems to indicate that this variation is in itself indicative of some definite change taking place in the organism, conceivably—for example—modifications in the reactions controlling discharge in the synapses. This would be consistent with the idea of assigning the value $\mu = 8{,}000 \pm$ to processes of central nervous origin determining breathing movements in the dogfish.

Another point may be made in relation to this matter of variation. When we speak here of " variation," we mean the tendency of such data to be scattered, in a seemingly random manner, in the band (Fig. 1) that we fit to them, and do not have reference to the error or mistake that the observer could have introduced involuntarily. The variation found in the data is *in* the material, truly a property of the organism, and not due to some lack of precision in the measurements. How could we in fact understand that the observer could make such errors that would cause all his data, after averaging, taking of reciprocals and logarithms, to fall in a band with parallel edges? A variation presenting such properties ought to permit us to analyze it as a function in itself. In other words we can consider, in the behavior of the organism, the behavior of a mathematical function and try to correlate one with the other. Analogous cases have been described by Crozier (1929).

First, why are the bands with parallel edges? It might be objected that it is impossible to conceive a regularity in the observations that would give such bands. It may be urged that viscosity, and the general complexity of protoplasm, would make the scatter of observational data rather irregular. One may point out that it would be astonishing if

in the very large number of cases now on record, such things as viscosity would have behaved consistently in a way that paralleled the behavior of the process considered; in other words, that viscosity and other properties should have a series of temperature characteristics always the same as that of the process under study. Furthermore, would it not be strange that from worms up to mammals the rule governing viscosity, for instance, would be similar to the one controlling respiration or cardiac rhythm? Is it not rather a case where the very inconsistent appearance of the data of some authors is indicative, more of the ways of operation of the observer than of some fundamental character so easily ascribed to protoplasm? One *could* advance several reasons why divergences from a band with parallel edges "should" be found, but in the absence of such effects they need not be considered.

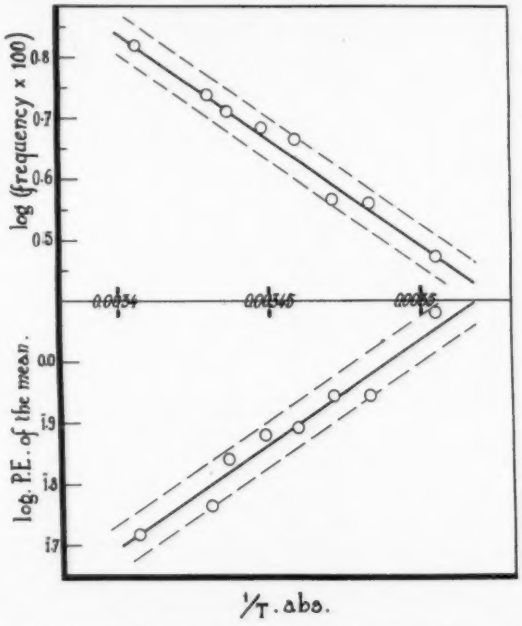

FIG. 2. The upper half of the graph is a reproduction of the points obtained for the animal represented by circles with horizontal diameters in Fig. 1. The lower half is a plot of the log of the P.E. of the mean frequencies of gill movements as a function of temperature. The slopes of the two lines are equal.

Let us go back to Fig. 1 and select out of the series of observations two sets to illustrate another point: a series (represented by a circle with an horizontal diameter) occurring in band *I*, and a second series

(indicated by solid circles) occurring in bands *I* and *II*. Each one of these series, plotted separately as in Figs. 2 and 3, shows this distribution of points in bands with parallel edges. We have represented also in the lower half of each figure the relation of the P.E. of the mean time for 20 beats to temperature for each of the points repre-

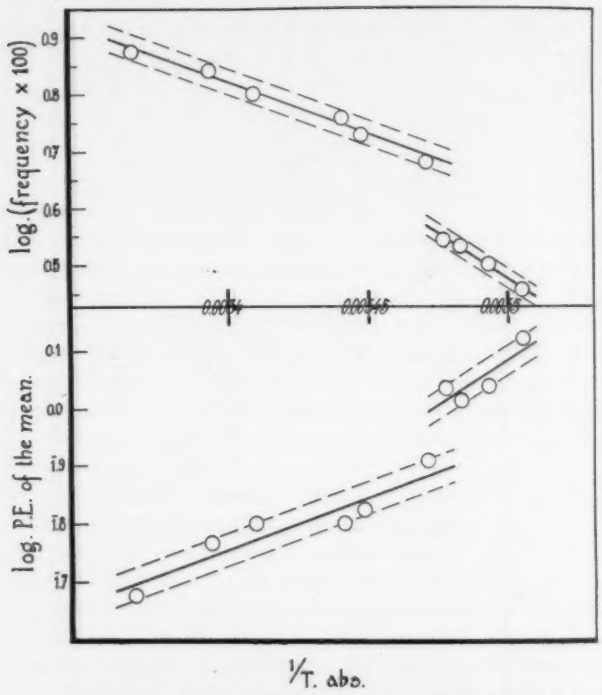

FIG. 3. The upper half of the figures is a reproduction of the points obtained for the animal represented by solid black circles in Fig. 1. The lower half is a plot of the log of the P.E. of the mean frequencies of gill movements as a function of temperature. The slopes of the lines are equal, and the break in each curve is located at the same temperature.

sented in the upper part. In each case the slope of the best-fitting line in the P.E. graph is the same as that obtained for the mean frequencies (*cf.* Crozier, 1929). In other words, the latitude of variation of the observations is a constant fraction of the mean, and both mean and variation of the mean are affected by temperature in the same way. Their temperature characteristics are the same. This is particularly illustrated in the second case where a definite shift from one value of

μ to another occurs. The P.E. curve shows the same break at the same point on the thermometric scale.

Two other conclusions can be drawn also: (1) a straight line relationship must exist between the P.E. of the mean (or the standard deviation of the mean) and the mean itself, (2) the standard deviation of the mean divided by the mean (*i.e.*, the coefficient of variability of the mean) must have no temperature characteristic (*i.e.*, the slope against 1/T must be zero. Each one of these conclusions has been illustrated in Figs. 4 and 5. [The use of σ_m (the standard deviation of the mean)

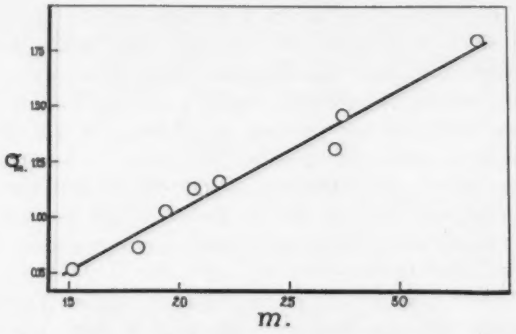

FIG. 4. The standard deviation of the mean frequency of gill movements is a linear function of the mean.

instead of the P.E. affects only the size of the unit and not the form of relation.] Obviously the straight line relationship is obeyed and the seemingly best-fitting line we draw through the scattered points of Fig. 5 is a line with a slope equal to zero or very close to it.

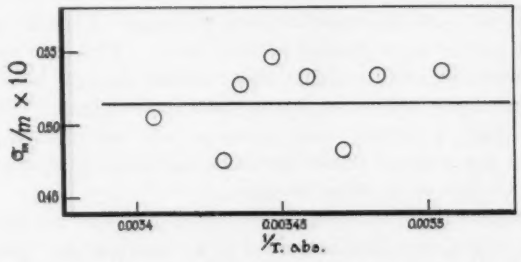

FIG. 5. The "variability" of the mean (*i.e.*, the standard deviation of the mean divided by the mean) as a function of temperature; this "variability" is constant.

An analogous case has been described by Crozier (1929, p. 95) in relation to the behavior of guinea pigs on an inclined plane. The angles of orientation assumed by the animals on the plane for different inclinations of the plane show a very marked change in their variation at a given point (45°). The probable error plotted versus the angle of tilt of the plane shows a marked break at 45°, much more marked indeed than the one in the regular plot of orientation versus tilt of plane. This change in a mathematical function can, here also, be definitely correlated with reactions in the animal: the type of creeping is different above and below 45°.

These observations lead to the conclusion that in the cases where experiments have been made in a controlled way, where averaging of data has been done with discrimination, and where, as a whole, the observations are correspondingly weighted, we can study not only the phenomenon itself, but also the laws of variation of this phenomenon. These provide an additional check on the existence of critical temperatures and enable us to fix them in a quite independent way. In such cases, the study of the P.E. and of the coefficient of variability will confirm for us the idea that biological material may not be so " variable " as it is often taken to be.

Observations were made also on the spiny dogfish (*Squalus acanthias*). These data are identical with those in Fig. 1 giving the μ value of 16,400. Here also, in a few instances we found sudden shifts to the value 35,000. As the observations were not numerous enough, they are not given in detail.

We seem to have here a case homologous with the one found by Crozier (1924) in the data of Miss Leitch (1916) on the rate of development of the radicle of *Pisum*. Crozier showed (*cf.* 1924, Fig. 13, p. 207) that in this case these increments can be found: one of 16,450 when the observations were taken over periods of 0.5 hour; a second and a third one (over different temperature ranges) when the determinations were carried over periods of 22.5 hours. This last series of increments was obtained for data which included thus the hours of darkness and the daily rhythm of cell division in the plants. The former, on the contrary, were taken over short periods which could thus " average " out any possible trend due to the moment of observation with respect to the normal division rhythm.

The possible coexistence of such a series points to the fact that any one of two (or three) factors can act at any moment as " pace maker," impressing thus its temperature characteristic on the whole system. Our case is homologous in that we also have found for different animals the shift of one increment to another indicating a corresponding shift

in the pace makers of the system. Any one of these could come into control and last for a certain time, and then be replaced by another one determining another thermal increment for the system.

Summary

The temperature characteristic or critical thermal increments (μ) for the movement of the spiracles and gill slits in the dogfish *Mustelus canis* (and *Squalus acanthias*) are 8,200; 16,400 and 35,000 calories. Critical temperatures are found at 14.5° C. and at 20.5° C. The latitude of variation of the measurements varies in a definite way and this is correlated with the occurrence of a critical temperature of 14.5° C.

The reactions controlling breathing movements seem to be catenary processes, one of which may be concerned with the central nervous control of the breathing movements, over a certain range of temperatures.

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HIBERNATION OF THE THIRTEEN-LINED GROUND SQUIRREL CITELLUS TRIDECEMLINEATUS (MITCHILL)

V. Food, Light, Confined Air, Precooling, Castration and Fatness in Relation to Production of Hibernation [1]

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Introduction

The cause of hibernation has been considered by many investigators and to account for it there has been presented a large number of theories, some based upon extended observations, others founded upon more or less casual studies of a small number of animals. In few instances have carefully controlled experiments been conducted. Most of these theories have been brought together by Rasmussen (1916).

Several authors admit that starvation tends to produce hibernation (Mangili, 1807; Hall, 1832; Merzbacher, 1904; Simpson, 1912; Mann, 1916; Shaw, 1925; and others). Most of these also state that little food was eaten shortly before a mammal goes into hibernation, indicating that hibernation took place when food was present and that starvation was not a factor. Dry food is considered a cause of æstivation (which is apparently a condition of lowered metabolism accompanied by a drop in body temperature, as in hibernation, but of a less degree and occurring in the summer or fall instead of in the winter) by Shaw (1925), Kashkarov and Lein (1927) and Kalaboukhov (1929).

Vitiated air was not considered as having any effect on hibernation in marmots by Mangili (1807), but Bert (1868, 1870) was able to produce lethargy in dormice by placing them at a low temperature in a bell jar deprived of air.

There appears to be an agreement among those authors who have mentioned adiposity that this condition is common in the fall among hibernators and that such a condition is favorable to hibernation, *e.g.*,

[1] Contribution 118 from the Department of Zoölogy, Kansas State Agricultural Experiment Station, Manhattan, Kansas. Earlier papers in this series deal with conditions in hibernation (Johnson, 1928), waking from hibernation (Johnson, 1929*a*, 1929*b*), and the influence of thyroxin, pituitrin and dessiccated thymus and thyroid on hibernation (Johnson and Hanawalt, 1930). For abstracts referring to portions of this work see Anat. Rec. 31: 337 and 37: 225.

Mangili (1807), Horvath (1881), Forel (1887*a*, 1887*b*), Mills (1892, 1893), Claparède (1905), and Mann (1916). According to Rasmussen (1916), this view was also presented by Sacc (1858).

No study has apparently been made relative to the effects of light and darkness, precooling, or castration, although Mann (1916) usually placed his ground squirrels in the dark in addition to starving them and cooling the room when he wished to produce hibernation.

The present work was begun in 1925 and has been continued to 1930 as time and numbers of animals available have permitted. Ground squirrels of the variety *Citellus tridecemlineatus pallidus* Allen from western Kansas were used. Since this variety has been broken up by Howell (Proc. Biol. Soc. Wash., 41: 211), it would seem that some at least of the animals used would belong to the southern group (*C. t. arenicola*) of what has previously been taken as all belonging to the variety or subspecies *C. t. pallidus*. Large numbers of ground squirrels could usually be obtained, so that the data of the different experiments could be treated statistically, thereby eliminating, to a large extent, personal judgment. Furthermore, for each animal placed under experimental conditions, another of like weight and sex was usually placed under conditions that were identical except for the one factor being tested, *e.g.*, food, confined air, etc. Observations soon showed that there was considerable individual variation and that the use of large numbers was necessary in order to make the results reliable. The work was done partly in a refrigeration room in which there was some daily fluctuation of temperature, but chiefly in an automatically controlled electric refrigerator in which there was very little fluctuation of temperature.

Experimental

Starvation and Darkness

These two conditions were studied together in nineteen experiments, from January to October, 1925. Each experiment lasted between 7 and 23 days. Three ground squirrels were fed in the light, three were fed in the dark, three were starved in the light, and three were starved in the dark. The starved animals were given water, green feed and, in most of the experiments, five kernels of corn daily. The fed animals were given water, green feed and liberal quantities of oats, with some wheat or corn at times. The light which was used to illuminate half of the cages was furnished by one 100-watt and one 50-watt electric bulb which were kept going throughout the experiments. The lights were at a distance of about six feet from the cages. The temperature of the cold room, which was approximately a ten-foot cube, showed an average range of daily fluctuation of 6° C.

At the end of an experiment the 12 animals were removed to the heated animal house and fed daily. Usually these were returned to the cold room in a new experiment in about two weeks and given treatment opposite to that in their previous period to balance out individual tendencies if there were any. It also avoided starving any animal at close intervals.

TABLE I

Food and light in relation to hibernation. Summary of daily records in the cold room.

The total days that the three animals were in the refrigeration room are given within the parentheses; the total days before hibernation for each group of three are given before the parentheses; the latter divided by the former gives the percentage of days before hibernation for each group of three animals and is given in the per cent column.

Date begun 1925	Time in days	Fed and light		Fed and dark		Starved and light		Starved and dark	
		Total days	Per cent	Total days	Per cent	Total days	Per cent	Total days	Per cent
Jan. 6	7	20 (21)	95	6 (21)	29	10 (21)	48	0 (21)	0
Jan. 14	9	16 (24)	67	9 (25)	36	5 (22)	23	5 (25)	20
Jan. 24	11	2 (33)	61	17 (33)	52	8 (33)	24	15 (33)	45
Feb. 4	12	14 (36)	39	25 (33)	76	7 (36)	19	5 (33)	15
Feb. 17	13	39 (39)	100	33 (39)	85	12 (39)	31	24 (39)	62
Mar. 3	14	42 (42)	100	42 (42)	100	11 (42)	21	12 (42)	29
Mar. 19	14	42 (42)	100	29 (39)	74	10 (42)	24	24 (26)	92
Apr. 2	16	48 (48)	100	42 (42)	100	7 (48)	15	6 (14)	43
May 7	23	56 (69)	81	69 (69)	100	34 (66)	52	66 (66)	100
May 30	15	45 (45)	100	45 (45)	100	23 (45)	51	7 (45)	16
June 16	18	54 (54)	100	43 (54)	80	31 (54)	57	30 (54)	56
July 4	13	39 (39)	100	37 (39)	95	26 (39)	67	24 (39)	62
July 18	13	26 (26)	100	39 (39)	100	10 (26)	38	29 (39)	74
Aug. 2	13	39 (39)	100	39 (39)	100	15 (39)	38	15 (39)	38
Aug. 15	13	27 (39)	69	27 (39)	69	3 (39)	8	11 (39)	28
Aug. 30	12	24 (24)	100	18 (36)	50	6 (24)	25	7 (24)	29
Sept. 11	11	7 (33)	21	15 (33)	45	23 (33)	70	14 (33)	42
Sept. 22	15	33 (45)	73	30 (45)	67	26 (45)	58	16 (24)	67
Oct. 8	16	34 (48)	71	9 (48)	19	3 (48)	6	17 (48)	35
Total days		607 (746)		574 (760)		270 (741)		327 (683)	
Average percentages		81.4		75.5		36.2		47.8	

In tabulating and evaluating the results (Table I) the number of animals given the same treatment (*e.g.*, given feed in the light) is multiplied by the days duration of the experiment (7 in the first experiment, to 23 in the ninth) to obtain the total days in the cold room, given in parentheses. The days spent in the cold room by each of the three animals before going into hibernation are added to obtain the

total days before hibernation, placed before the parentheses. The latter divided by the former gives the percentage of days before hibernation. The totals of the experiments are brought together in Table II and the percentage of days before hibernation computed for each of the four conditions, fed and light, fed and dark, starved and light, and starved and dark. When the percentages of days before hibernation are compared, it is observed that both of the fed groups averaged much higher than the starved groups. Feeding therefore delays hibernation, or starvation hastens entrance into this condition. The two "dark" groups show no marked difference from their corresponding "light" groups. Light or darkness appears to play very little, if any, part, then, in inducing hibernation.

TABLE II

Summary of Percentages of Days Before Hibernation from Table I

	Fed vs. Starved			Light vs. Dark	
Light	81.4	36.2	Fed	81.4	75.5
Dark	75.5	47.8	Starved	36.2	47.8
Averages	78.5	42.0	Averages	58.8	61.6

Summary of Percentages of Days in Hibernation from a Table Not Published

	Fed vs. Starved			Light vs. Dark	
Light	10.9	48.0	Fed	10.9	15.2
Dark	15.2	36.8	Starved	48.0	36.8
Averages	13.0	42.4	Averages	29.4	26.0

The data have similarly been tabulated to show the percentage of days in hibernation as a different measure of the production of hibernation. Only the summary of this table is included in Table II. The average percentage of days in hibernation is much higher for the starved groups than for the corresponding fed groups, showing in a different way the effectiveness of starvation in the production of hibernation. Here also there is no marked difference between the two dark and the two light groups. The difference is still less when the average of the two light groups is compared with that of the two dark groups. Statistical treatment showed that the results between each fed group and its corresponding control was significant (Difference/Probable Error = 11.3 in one case and 6.3 in the other case). The difference

between the "light" and "dark" groups was not significant (D/PE = 1.6 and 2.4, respectively).

It appears safe to conclude from these experiments that starvation is a condition favorable to the production of hibernation, whereas darkness is no more conducive to hibernation than artificial light of a strength such as is used in an ordinary well-lighted room. Whether ultra-violet light might stimulate the animals to stay awake has not been investigated.

Confined Air

Preliminary experiments in which some animals had been placed in half-gallon tin cans with tight lids in which were punched only two six-penny nail holes about 3.0 mm. in diameter resulted in death in some of the animals. Four such holes, however, appeared to admit sufficient air to prevent suffocation at refrigerator temperatures. In each experiment three animals were placed in the four-hole cans, three were placed in open wire cages (6 x 6 x 10 inches) as controls and three others were placed in half-gallon cans with highly perforated lids to admit an abundance of air. The latter controls were used to determine the effect, if any, of the limited space on the experimental (confined air) animals. Except in a few short preliminary experiments the animals were fed whole oats, wheat or corn, and a small amount of green grass or sprouted oats daily. Wood shavings were used as nest material and in the cans also served to absorb the moisture from the breath of the animal. Each experiment was usually terminated after 7 days. The animals were usually undisturbed except for the daily observations. All the animals were in the dark.

From Table III it is to be noted that in all four sets of experiments or periods the confined air animals went into hibernation sooner and stayed in more of the time than the animals in the open cages. A summary of the results of these experiments shows that those animals in confined air went into hibernation after 19 per cent of the total days they were in the refrigerator as compared to 49 per cent in the case of the open cage animals. These results are quite significant (D/PE = 9.5) in showing the retarding effect of free air upon entrance into hibernation. The confined air animals were in hibernation 68 per cent of the days in the refrigerator, whereas those in open cages were in hibernation only 41 per cent of the days. These results are also significant (D/PE = 9.4). The total differences of periods 1 and 2 (Table III) and also of periods 1, 2 and 3 were found to be significant.

The confined air animals also showed an earlier entrance into hibernation and more days in hibernation than did those in the cans with

TABLE III

Results of Confined Air Experiments

Period	Experimentals in Cans with four holes			Controls in cans with many holes			Controls in open wire cages		
	No. of Animals	Per cent of Days		No. of Animals	Per cent of Days		No. of Animals	Per cent of Days	
		Before hibernation	*In* hibernation		*Before* hibernation	*In* hibernation		*Before* hibernation	*In* hibernation
April 1925 to June 1925	18	15	74	—	—	—	16	81	15
Nov. 1925 to June 1927	50	20	66	51	33	46	51	39	49
June 1927 to Oct. 1927	27	14	73	25	55	37	25	52	38
Sept. 1929 to Feb. 1930	13	26	59	15	31	56	15	42	48
Total Period* April 1925 to Feb. 1930	108	19	68				108	49	41
Period* Nov. 1925 to Feb. 1930	90	19	67	91	39	45	91	43	45

* Owing to the difference in number of animals in the groups above, these percentages are not averaged from the above averages, but from a table which contains the percentages for each animal.

many holes. The percentage of days before hibernation was 19 in the case of the experimental and 39 in the case of the controls in the many-hole cans (D/PE = 5). The percentage of days in hibernation was 67 for the experimentals and only 45 for the controls in many-hole cans (D/PE = 6.7). These results show a significant increase in early entrance into hibernation and continuance in this state because of the confined air, and show that the limited space of the cans played little, if any, part in the production of hibernation in the four-hole cans. It is to be noted, however, that in the last experiments (13–15 animals) nearly as much hibernation occurred in the cans with many holes as in those with four holes. In the other groups of experiments which included larger numbers of animals this was not the case. In these last experiments two conditions were unfavorable to hibernation in the four-hole cans. One was that the refrigerator was running very little and did not freeze the condensed moisture in the refrigerator as in active refrigeration, resulting in a high humidity in the cans. The other was that the animals were placed in the four-hole cans just as soon as placed in the refrigerator. Furthermore, the temperature in the refrigerator was not as low as usual. In this connection it should be stated that one condition tending to retard hibernation in the four-hole cans, in most of the experiments, was the retention of heat in these cans as long as the animal was active, *i.e.*, not torpid. Observations showed the temperature to be higher in these cans than in the cans with many holes. In some cases the animal appeared to be irritated by the confined air if it did not soon become torpid, and in the last series of experiments some of the confined air animals died.

Two methods of lowering the temperature in the four-hole cans were tried. In one experiment these cans were placed on a shelf below the others which was not over one degree cooler. In three other experiments the four-hole cans were wrapped on the sides by a small towel and then set in a shallow pan of water. Evaporation tended to cool the can one to three degrees below the temperature of the cans with many holes when the animals were normal in both. To avoid overcooling the four-hole cans, it was deemed best to place them on the same shelf without additional cooling by wet towels, etc., and permit the higher temperature to be a handicap to hibernation. Confined air, therefore, is probably more effective in the production of hibernation than indicated by these experiments and doubtless aids production of hibernation in nature, since the animals plug the entrance into their burrows when they retire in the fall. The limited space played very little part in the production of hibernation, possibly because the animals

usually roll up in the nest whether in a large or small cage except when eating. Most of these animals did not actively try to escape from the cages or cans.

Precooling

Proponents of the view that hibernation is produced not by external conditions, but by an internal seasonal rhythm, have pointed to the greater difficulty in producing hibernation in the summer with artificial cold than in winter. While less tendency to hibernate in the spring and early summer than in the early winter has been noted by the author, it must be recognized that several external conditions may be different in the fall from those obtaining in the spring and summer. The effects of a previous season of hibernation, the poor condition of the wild animal when caught for early summer study, and the greater excitability of new wild animals also usually caught just before the summer experiments are in contrast with the conditions produced by keeping the animals one to several months for the study of winter hibernation. The observations made so far suggest that these conditions influence hibernation. In the summer, also, animals are likely to be removed directly from a warm room into a refrigerator, whereas in the fall they are often taken from a room which has a low and possibly fluctuating temperature. This precooling of the animal nightly in an animal house is almost unavoidable unless special pains are taken to control the temperature. Drops in room temperature to 60° or even 40° C would not be unusual under ordinary conditions of heating.

The effect of this condition of daily precooling has been investigated in seven experiments. In each experiment 10 animals were taken to a cold cave or attic where the temperature averaged about 16° or 17° C. for an average of about eight hours in one experiment and 15 hours in the others for a period of about ten days. Ten controls were kept in the animal house at an average temperature of about 26° C. or about 80° F. The animals in the cave or attic occasionally became partly torpid, but controls in the animal house did not hibernate there.

After this preliminary treatment, the animals were all removed to a refrigerator kept at a temperature which was constant or changed very slowly. The average temperature was about 7 or 8° C. Experimental (precooled) and control animals were on the same shelf, and therefore under identical conditions. In the seven experiments (Table IV) a total of 62 precooled animals went into hibernation after an average of 35.5 per cent of their days in the refrigerator while the controls entered hibernation only after 60.5 per cent of their total days in the

refrigerator, indicating that precooling caused the animals to hibernate sooner. In the per cent of days in hibernation the effect of precooling was also evident, but not to the same extent. The precooled animals

TABLE IV

Precooling Experiments

Date	Precooled (Experimental) Animals				Controls		
	No. of animals	Per cent days before hibernation	Per cent days in hibernation	No. of days	No. of animals	Per cent days before hibernation	Per cent days in hibernation
1927							
Jan. 1	9	34	43	15	9	43	29
Jan. 12	10	42	44	13	10	75	20
Jan. 27	7	29	40	15	8	72	15
Feb. 15	10	41	30*	14	10	90	8*
1928							
Mar. 6	7	41	24*	8	9	72	9*
1929							
Nov. 7	9	35	43†	11	9	29	51†
Nov. 16	10	20	63	6	9	36	48
Averages‡		35.5	41.7			60.5	26.0
Below are those experiments in which less than three animals in either group (experimental or control) hibernated.							
1927							
Mar. 2	9	81	8	14	10	93	3
Apr. 3	9	93	2	18	10	84	13
Apr. 27	6	100	0	7	6	100	0
1930							
Feb. 4	9	98	2	7	9	70	14

* Note low percentage of hibernation in February and March, yet the precooled animals entered hibernation much sooner than the controls.

† Both groups went into hibernation very quickly in this experiment. Controls had been partly precooled, as the room temperature fell to 21° C. or 13° C. each night. Experimentals were in a temperature over night which fell to 10–20° C.

‡ Averages from another table in which each animal is counted equally. Not an average of the group averages given above.

hibernated 41.7 per cent of their total days in the refrigerator, while the controls hibernated only 26.0 per cent.

The difference in days *before* hibernation of the two groups was quite significant (D/PE = 6.48) and that for days *in* hibernation was fairly significant (D/PE = 3.88). As the influence of the precooling would wear off in a few days after placing the animals in the refrigerator, more importance should be attached to the data of days *before* hibernation than those of days *in* hibernation.

It should be noted that precooling was conducive to increased hibernation in the months of November to January, with one exception in which the controls had been precooled slightly through inability to control the temperature of the animal house. Precooling was less or not at all effective in the late winter and spring months, the period in which the animals gave indications of increased sexual activity by enlargement of the testes, scrotum and penis in the male and by enlargement of the vulva in the female. While exact comparisons are difficult to make between hibernation at the different seasons, it may be stated that observations over a period of years indicate that spring is not as good a season for hibernation as is the summer.

These experiments would indicate that one cause of more hibernation in the winter than in summer in the laboratory is sometimes a physiological condition produced by external conditions such as intermittent cold which aids in the production of hibernation, providing other conditions are moderately favorable to entrance into hibernation. Intermittent cold may not play a great part in nature, since the daily range is not great between six inches and one foot in the ground (Bouyoucos, 1913, 1916; Sweezy, 1903, McColloch and Hayes, 1923), where most of the hibernating nests occur (Johnson, 1917). However, a daily range of 10° F. or more was frequently noted at the six-inch level by Bouyoucos (1916) in loam and clay in September, but the range was only about half as great in October, when the soil temperature was falling gradually. It seems probable, however, that the gradual cooling of the soil would have a similar effect to that of intermittent precooling in that it would prepare the animal for hibernation. Whether precooling or gradual cooling would eliminate mortality in hibernation in summer cannot now be stated, but general observations point in that direction. It should also be noted that Payne (1927) has found that preliminary cooling as well as dehydration enables insects to withstand more severe and longer periods of cold.

Castration

Since ground squirrels become sexually active very soon after they come out of hibernation and in the animal house begin to show enlargement of the external genitalia in late winter, it was thought that

waking from hibernation in the spring was possibly induced to some extent by the increasing activity and internal secretion of the gonads. To test this possibility, a number of male ground squirrels were castrated and after a time were placed in a refrigerator with uncastrated controls. Some animals were used several times. Each experiment usually lasted two weeks. In the first four experiments, consisting of 3 to 5 castrated animals with the same number of controls, from May to July, 1926, there was no hibernation in either castrated or control (uncastrated) animals. From July to December, 1926, five more

TABLE V

Effect of Castration upon Hibernation in Late Winter and Early Spring

No. of animals	Experimental (castrated) male ground squirrels				Control (uncastrated) male ground squirrels		
	Per Cent days before hibernation	Per Cent days in hibernation	Date begun	Days in refrigerator	No. of animals	Per Cent days before hibernation	Per Cent days in hibernation
			1927				
6		52	Jan. 13	28†	6		43
6		82	Feb. 11	13†	6		37
4		36	Feb. 24	70†	4		13
4	42	43	Mar. 22	50	4	92	6
			1928				
3	0	78	Jan. 30	15	2	25	51
2	32	47	Mar. 8	19	1	28	51
1	100‡	0	Apr. 16	12	1	100‡	0
Av. 2.5	44	42		24	2	61	27
Average of individual records	27.6	55.6				46.2	33.6

† Continuation of a previous experiment.
‡ Animals thin, a condition tending to prevent hibernation.

experiments, and in August and September, 1927, two more experiments were performed in which there was an average of 34.5 per cent of days in hibernation in the 48 castrated males and 35.3 in the 46 uncastrated males, showing no effect of castration on hibernation in the summer, fall, and early winter.

Experiments performed from January to March, 1927, and January to April, 1928, showed an inhibitory effect of the gonads upon hibernation at this time of year (Table V). The castrated males showed 55.6 per cent days in hibernation, whereas the controls showed only 33.6 per cent days in hibernation in this experiment. The difference

is probably significant (D/PE = 3.52) because in other experiments normal males have not hibernated much at this time of the year and because a castration experiment with females at this time of year resulted similarly. In five experiments (January to March), with an average of 3 animals in each, the spayed females hibernated 45 per cent and the normal females only 30 per cent of the days in the re-

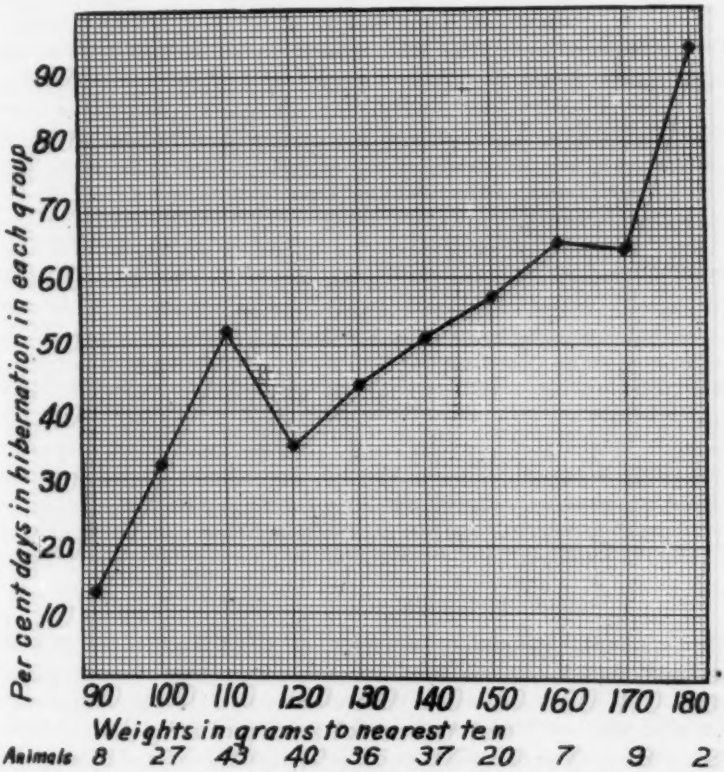

FIG. 1. Graph showing relation of weight to tendency to hibernate in ground squirrels. The bottom row of figures gives the number of animals having the weight given directly above.

frigerator. The spayed animals were in the refrigerator 34 per cent of the total days in the refrigerator before they hibernated whereas the controls were in the refrigerator 45 per cent of the total days before they hibernated. As the number of females is small and the difference not so striking as in the case of the males, these data were not treated

statistically. They lend support, however, to the data on the males as the results point in the same direction. It is not improbable that the increased sexual activity which is apparently in part responsible for lack of hibernation in spring is in turn caused by increased activity of the anterior pituitary. Work (unpublished) on the relation of this gland to hibernation has been in progress at this laboratory since 1926.

Weight and Hibernation

As weights were taken before and after each experiment in the refrigerator, it was possible to use existing data for a determination of the relationship between weight and hibernation. From these data the graph in Fig. 1 was made. Thirty-eight heavy animals weighing between 146 and 185 grams hibernated 62 per cent of the total days in the refrigerator, while 118 light animals weighing between 86 and 125 grams hibernated only 39 per cent of the total days in the refrigerator. Treated statistically, this is a significant difference (D/PE = 5.7). The number of days before hibernation took place was only 16 per cent of the total days in the refrigerator in the case of the heavier animals but was 41 per cent in the case of the leaner animals. This difference is also significant (D/PE = 8.1). It is to be seen, however, that there was not a perfect gradation of per cent of days of hibernation (Fig. 1) from the lightest to the heaviest animals; thus 43 animals weighing between 106 and 115 grams (given as 110 grams in the graph) hibernated more than another group of 40 animals weighing 10 grams more, but a predominance of high percentages of days *in* hibernation is found in the heavier groups and there is a predominance of high percentages of days *before* hibernation in the lighter groups.

Summary

Controlled experiments performed on large numbers of ground squirrels showed that individuals starved or given very limited rations went into hibernation sooner and hibernated longer than those receiving an abundance of food. Moderately bright illumination with electric light did not affect the tendency to hibernate in any way. Confined air and precooling the animals nightly both aid in the production of hibernation. Castration does not influence hibernation except during the breeding season in the spring when castrated males hibernate more readily than normal males. The few spayed females used also hibernated more than the normal females in the spring. Obesity is favorable to hibernation.

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TIME OF DEVELOPMENT OF THE DIFFERENT SEXUAL FORMS IN *DROSOPHILA MELANOGASTER*

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In the progeny of a triploid female crossed to a normal diploid male there appear six different sexual types. These are the diploid females and males, triploid females, intersexes, and the two kinds of so-called supersexes, namely superfemales and supermales. As shown by Bridges (1921, 1922) these sexual types are due to variations in the ratio between the number of X-chromosomes and the number of sets of autosomes present in cells of a given individual. According to Bridges the different sexual types have the following ratios:

	Number of X-chromosomes	Number of Sets of Autosomes	Ratio
Superfemale	3	2	1.50
Diploid female	2	2	1.00
Triploid female	3	3	1.00
Intersex	2	3	.67
Male	1	2	.50
Supermale	1	3	.33

Most of the characters of the flies change in this series of sexual types hand in hand with the change of the sex-determining ratio. There are, however, some characters of the flies which seem to be independent of the sex-determining ratio. Among such characters may be listed the size of the cells, which is correlated with the amount of chromosomal material present in their nuclei (Dobzhansky, 1929). Another character of this kind is the length of the development period, to the study of which the present paper is devoted.

The length of the development period has been studied previously in only the diploid males and females (Bonnier, 1926). According to Bonnier, the development of males takes a slightly longer time than that of females. At 25° the development of a female takes 227.98 hours on the average, and the development of a male 232.24 hours. At 30° the corresponding figures are 178.10 and 187.63 hours.

The technique of the present study was as follows. Batches of from twenty to thirty wild-type triploid females were kept together

with an approximately equal number of males for two days to ensure fertilization. The flies were then transferred to the ordinary culture-bottles provided with a standard amount of food, but without filter-paper, which is regularly placed in each culture-bottle. Here the flies were allowed to lay eggs during a two-hour period, and after that were again transferred to fresh bottles without paper, and again allowed to lay eggs during another two-hour period. The above procedure was repeated from five to six times with each batch of flies. All the transfers from bottle to bottle were performed without etherization of the flies, because females of *Drosophila* are known to interrupt the egg-laying for several hours after being etherized. The bottles with eggs secured by this method were placed in an incubator and kept at 27°. As soon as the mature flies began to emerge, bottles were examined every two hours. In young flies the determination of the sexual type is sometimes difficult; in cases of doubt, flies were kept alive until the sex could be determined with certainty. Only those bottles which produced more than 20 but less than 60 flies were taken into account (the average number of flies per bottle was 38.5). All other bottles were

TABLE I

Time of Emerging from the Pupæ

Time in Hours	Diploid Females	Triploid Females	Males	Super-females	Inter-sexes	Super-males
183	1	—	—	—	—	—
185	14	2	—	—	—	—
187	19	3	2	—	—	—
189	16	4	2	—	—	—
191	35	6	6	—	—	—
193	25	3	2	—	—	—
195	23	—	5	—	—	—
197	25	2	2	—	—	—
199	26	—	9	—	—	—
201	29	5	8	—	—	—
203	29	2	4	—	—	—
205	29	7	26	—	—	—
207	21	6	10	—	—	—
209	22	11	9	—	—	—
211	7	7	7	—	—	—
213	7	6	5	—	—	—
215	4	1	2	—	—	—
217	1	—	1	—	—	—
219	1	—	2	—	—	—
221	—	—	—	—	—	—
223	—	—	—	—	1	—
225	1	1	—	—	1	—
227	—	2	—	—	—	—

TABLE I (*Continued*)

Time in Hours	Diploid Females	Triploid Females	Males	Super-females	Inter-sexes	Super-males
229	1	—	—	—	2	—
231	—	—	—	—	1	—
233	—	—	1	—	3	—
235	5	1	3	—	5	—
237	—	—	—	—	2	—
239	—	—	—	—	13	—
241	—	—	—	—	12	—
243	—	—	—	—	10	—
245	—	—	—	—	8	—
247	—	—	—	—	7	—
249	—	—	—	1	6	—
251	—	—	1	—	9	1
253	—	—	—	1	12	—
255	—	—	—	—	24	—
257	—	—	—	—	18	2
259	—	—	—	—	22	—
261	—	—	—	—	20	1
263	—	—	—	—	10	1
265	—	—	—	—	11	1
267	—	—	—	—	5	1
269	—	—	—	—	4	—
271	—	—	—	—	3	2
273	—	—	—	—	3	1
275	—	—	—	—	2	1
277	—	—	—	—	2	1
279	—	—	—	—	4	—
281	—	—	—	—	6	—
283	—	—	—	—	4	—
285	—	—	—	—	1	—
287	—	—	—	—	—	3
289	—	—	—	—	2	1
291	—	—	—	—	1	—
293	—	—	—	—	—	—
295	—	—	—	—	—	—
297	—	—	—	—	—	1
Total	341	69	107	2	234	17

discarded, since overcrowding of food with larvæ might slow down the development, especially of the weaker types, and bottles producing too few flies usually have the food in poor condition.

The technique just described gives a reasonable degree of certainty that all the flies developed under similar external conditions and that the length of the development period of each individual fly is determined with an error not exceeding $\pm$ 1 hour. The material gathered in this way is presented in Table I. As seen from this table, 770 flies were obtained. Among them there were 44.3 per cent diploid females,

9.0 per cent triploid females, 13.9 per cent males, 30.4 per cent intersexes, 0.3 per cent superfemales and 2.2 per cent supermales. This is about the normal frequency of the different sexual types in the progeny of triploid females. In another experiment, in which offspring were obtained from individual triploid females kept in the bottles until their progeny appeared, there were 50.8 per cent diploid females, 6.4 per cent triploid females, 7.9 per cent males, 32.3 per cent intersexes, 0.2 per cent superfemales and 2.4 per cent supermales. (The total number of flies in this experiment equals 8796 flies; the average number of flies per bottle equals 71.6).

Consideration of the data presented in Table I shows at once that the different sexual types do not develop equally fast. The six known

TABLE II

The Length of the Development Period (in hours) of the Different Sexual Types

	Mean Value	σ	C	n
Diploid females	199.24 ± .49	9.12	4.5	341
Triploid females	203.83 ± 1.29	10.72	5.3	69
Males	205.20 ± .98	10.08	4.9	107
Intersexes	255.76 ± .82	12.52	4.9	234
Superfemales	251.00	—	—	2
Supermales	272.72 ± 3.12	12.90	4.7	17

types may be divided roughly into two groups. The development of the diploid and triploid females and males takes from 185 to 219 hours, and only a few individuals require longer than that period. On the other hand, intersexes, superfemales and supermales begin to hatch after 220 hours. That is to say, there exists a short period of time when scarcely any flies emerge in the cultures.

The results of the statistical treatment of the material presented in Table I are shown in Table II. The development of the diploid females takes the shortest time as compared with other sexual types. The development of males takes 5.96 ± 1.05 hours longer than that of the diploid females. This difference is statistically significant and is approximately equal to that obtained by Bonnier in his experiments. The absolute values for the length of the development of diploid females and males are also in good agreement with Bonnier's data (one must, of course, take into account that the temperature used in the present experiments is intermediate between the two temperatures used by Bonnier).

The figure obtained for the length of the development of triploid

females is intermediate between the figures obtained for diploid females and males, but the differences are not statistically significant in either case. It may be concluded that the development of the triploid females takes approximately as much time as the development of the diploid females and males, and far less than the development of the intersexes and the supersexes.

The development of the intersexes takes 255.76 ± .82 hours on the average. This figure is unquestionably different from those obtained for females and males. Although the figure for the superfemales (251 hours) is based only on two individuals, it may be taken for certain that the length of the development of the superfemales is of the same order of magnitude as that of the intersexes and the supermales. This has been proved in a different experiment in which superfemales were obtained in greater number together with diploid females and males (this experiment deals with the progeny of attached-X females crossed to normal males). In this experiment superfemales appeared in the bottles only in the later counts, when the number of diploid females and males hatching in a given interval of time was already declining. The length of the development of the supermales (Table II) is significantly greater than that of the intersexes. That is to say, the supermales develop more slowly than any of the other sexual types known.

It may be concluded that the length of time of development does not show a correlation with the ratio between the number of X-chromosomes and the number of sets of autosomes, the ratio which is known to determine the sexual type. The types studied may be divided into two groups: the balanced and the unbalanced types. The balanced types are those which have the sex-determining ratio equal to that observed in diploid females or males. Beside the diploid females and males only the triploid females belong to this group. Their development takes a relatively short time. The unbalanced types are the intersexes and the supersexes; their development takes a longer time.

Summary

1. The different sexual types known in *Drosophila* are characterized by different lengths of their development periods. Diploid females develop more quickly and supermales develop more slowly than the other sexual types, which are intermediate between these two extremes (see Table II).

2. There is observed no correlation between the length of development and the sex-determining ratio (*i.e.*, the ratio between the number of X-chromosomes and the number of sets of autosomes present in the cells of a given individual).

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